# Molecular genetics of interactions between *Xanthomonas campestris* pv. *raphani* and *Arabidopsis thaliana*

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# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# LIST OF ABBREVIATIONS

| | |
|---|---|
| **A** | Absorbance |
| **aa** | Amino acid |
| **AGI** | Arabidopsis Genome Initiative |
| **AIMS** | Arabidopsis Information Management System |
| **ATCC** | American Type Culture Collection, Manassas, USA |
| **Avr** | Avirulence |
| **BLAST** | Basic Local Alignment Search Tool |
| **bp** | Base pair |
| **CFBP** | French Collection of Plant associated Bacteria, INRA, Angers, France |
| **cfu** | Colony-forming unit |
| **cM** | Centimorgan |
| **cv.** | Cultivar |
| **dai** | Days after inoculation |
| **DNA** | Deoxyribonucleic acid |
| **HRI** | Formerly Horticulture Research International, now University of Warwick Crop Centre, Wellesbourne, UK |
| **ICMP** | International Collection of Microorganisms from Plants, Lincoln, New Zealand |
| **IP** | Interaction phenotype |
| **kb** | Kilo base pair |
| **LB** | Luria-Bertani |
| **LMG** | Laboratory for Microbiology, Faculty of Sciences, Ghent University, Ghent, Belgium |
| **LOD** | Logarithm of the odds ratio |
| **Log** | Logarithm base 10 |
| **Mb** | Mega base pair |
| **NCBI** | National Centre for Biotechnology Information, USA |
| **NCPPB** | National Collection of Plant Pathogenic Bacteria, Fera, York, UK |
| **nt** | Nucleotide |
| **PCR** | Polymerase chain reaction |
| **Pers. comm**. | Personal communication |

| | |
|---|---|
| **pv.** | Pathovar |
| **R** | Resistance |
| **RefSeq** | Reference sequence database of the National Centre for Biotechnology Information, USA |
| **RIL** | Recombinant inbred line |
| **subsp.** | Subspecies |
| **SNP** | Single nucleotide polymorphism |
| **sp.** | Species |
| **syn.** | Synonym |
| **TAIR** | The Arabidopsis Information Resource |
| **T3SS** | Type III secretion system |
| **T3E** | Type III effector |
| **USDA-ARS** | United States Department of Agriculture - Agriculture Research Service |
| **UV** | Ultraviolet |
| **var.** | Variety |
| ***Xcc*** | *Xanthomonas campestris* pv. *campestris* |
| ***Xcr*** | *Xanthomonas campestris* pv. *raphani* |
| **YDC** | Yeast dextrose calcium carbonate |

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# DECLARATION

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. The work presented (including data generated and data analysis) was carried out by myself except in the cases outlined in the relevant parts of the text.

# SUMMARY

The major aim of this research was to investigate interactions between the bacterial pathogen *Xanthomonas campestris* pv. *raphani* (*Xcr*) and *Arabidopsis thaliana* which are largely unexplored as a model pathosystem, and to identify genetic loci conferring resistance to this pathogen. *Xcr* is genetically close to *X. campestris* pv. *campestris* (*Xcc*) and both pathogens infect common *Brassicaceae* species including *A. thaliana*, but cause distinct diseases - leaf spot and black rot, respectively.

Phenotypic variation was identified among interactions between 22 *A. thaliana* accessions of wide geographic origin and *Xcr* and *Xcc* strains representing known host genotype specific races. Accessions were identified showing broad resistance and broad susceptibility to multiple *Xcr* and *Xcc* strains as well as accessions that differentiate between *Xcr* races.

Genetic mapping of resistance to a strain of *Xcr* race 2 in a multi-parent recombinant inbred population revealed two major effect loci: *RXCR1* at the bottom arm of chromosome 3, and *RXCR2* at the bottom arm of chromosome 5. *RXCR1* was confirmed by fine-mapping and loss- and gain-of-function experiments, as a single gene encoding a kinase-like protein conferring resistance in the accession Columbia. Columbia is resistant to strains of all three known *Xcr* races, but *RXCR1* is insufficient on its own to explain the broad resistance. A summary of phenotypic and genetic analyses of interactions between *Xcr* and *A. thaliana* is presented in a gene-for-gene model.

*X. campestris* strains associated with outbreaks of a leaf spot and blight disease of brassica crops in Mauritius, were characterized. These strains were similar to reference *Xcc* strains in pathogenicity tests and molecular analyses. The presence of *Xcr* in these outbreaks was not confirmed.

Whole-genome sequencing data was used to identify genes that may contribute to the distinct modes of pathogenesis of *Xcr* and *Xcc* as well as variation in host specific races within each pathovar. Genes differentially present/absent between nine *Xcr* and 23 *Xcc* strains were identified and include genes predicted/known to encode type III effectors some of which have been previously described to have an effect on *Xcc* pathogenicity. Candidate avirulence determinants of *Xcr* and *Xcc* races were also identified.

# CHAPTER 1. General introduction

Plant pathogens are a major problem worldwide. They cause crop losses and have important economic, social and environmental impacts, affecting directly or indirectly every one of us. Throughout human history, plant pathogens have caused devastating epidemics, and continue to affect 'one or more of the prerequisites for a healthy, safe life for humans' (Agrios, 2005). They pose serious threats to the world food security and contribute to depletion of important natural resources such as water and arable land (Agrios, 2005; Strange & Scott, 2005). At least 14% of global crop production is lost due to plant pathogens alone and cost more than $200 billion annually (Agrios, 2005; Popp & Hantos, 2011). In a world of limited resources with an expanding global population, the impacts of plant pathogens urge efforts for deployment of sustainable and efficient crop protection strategies.

Plant pathogens include diverse organisms such as bacteria, fungi, oomycetes and nematodes, and establish complex interactions with plants that are still not completely understood. They invade and colonize the plant host by releasing toxins, enzymes and other molecules that continuously interfere with the host structural integrity, metabolism and inducible defence responses leading to an abnormal plant condition described as disease. In turn, plants may halt invasion by potential pathogens through pre-existing or activated defence mechanisms that include structural barriers and diverse biochemical reactions that produce antimicrobial compounds or create conditions for inhibiting growth and spread of pathogens (Agrios, 2005). The study of the complex interactions between diverse plant pathogens and their hosts as well as other equally important biotic and abiotic factors that influence these interactions, is providing and will continue to provide valuable knowledge to improve disease management (Wilkinson *et al.*, 2011; Reid & Greene, 2012; Dangl *et al.*, 2013).

Bacterial pathogens in particular, can cause severe crop losses in the field or during storage, and deprive production of certain crops where they remain a threat. They are difficult to control, effective curative measures are scarce and disease control often relies in preventive measures and eradication of infected plants (Agrios, 2005; Beattie, 2006). Phytopathogenic bacteria are distributed across 161 species and 36 genera (Bull *et al.*, 2012; Bull *et al.*, 2014) including genera of economic and scientific importance such as *Agrobacterium*, *Clavibacter*, *Erwinia*, *Pseudomonas*, *Ralstonia*, *Xanthomonas* and *Xylella* (Mansfield *et al.*, 2012).

The *Xanthomonas* genus includes diverse phytopathogenic bacteria that collectively cause disease in more than 350 plant species (Leyns *et al.*, 1984) and have been reported to cause severe losses in several important crops (Hayward, 1993; Brown, 2001; Das, 2003; Quezado-Duval *et al.*, 2004; Strange & Scott, 2005; Wechter *et al.*, 2008; Tripathi *et al.*, 2009). Among phytopathogenic xanthomonads, the pathogen *X. campestris* pv. *raphani* (*Xcr*) has received little attention. It causes a leaf spot disease in a wide range of plants that includes economically important crops such as brassicas, radish, tomato and pepper, and it has been identified in several countries (Tamura *et al*., 1994; Vicente *et al.*, 2006). *Xcr* is genetically close to one of the most important pathogens of brassica crops worldwide – *X. campestris* pv. *campestris*, (*Xcc*) (Williams, 1980; Vauterin *et al.*, 1995; Vicente *et al.*, 2006; Fargier *et al.*, 2011). Both pathogens affect some common hosts (brassicas and radish), but they colonize preferentially different host tissues and cause distinct disease symptoms (White, 1930; Tamura *et al.*, 1994; Vicente *et al.*, 2006; Fargier & Manceau, 2007).

Thus, the overall aim of the present study was to further investigate *Xcr* in comparison to *Xcc*. Specifically, it aimed to characterize the phenotypic variation of *Arabidopsis thaliana* in response to different races of *Xcr,* to identify genetic loci controlling resistance in this model plant species to at least one race of *Xcr*, and to use comparative analysis of genomes of both pathovars to identify candidate determinants of their distinct modes of pathogenesis and avirulence of races defined within each pathovar.

## 1.1 *Xanthomonas*, a genus of diverse bacterial plant pathogens

The genus *Xanthomonas* comprises Gram-negative bacteria that are typically rod shaped, motile by a single polar flagellum and are obligate aerobes (Saddler & Bradbury, 2005). Bacterial colonies are usually yellow pigmented due to the presence of brominated aryl polyenes (or xanthomonadins) in the outer-membrane of the bacterial cell walls (Saddler & Bradbury, 2005), which might confer protection against photobiological damage (Jenkins & Starr, 1982; Rajagopal *et al.*, 1997; Poplawsky *et al.*, 2000). Most strains also produce xanthan which is an extracellular polysaccharide that confers a viscous consistency to bacterial colonies and plays an

important role in bacterial survival and colonization of plants (Rudolph, 1993; Saddler & Bradbury, 2005; Meyer & Bogdanove, 2009).

Although non-pathogenic xanthomonads have been identified in association with plants (Vauterin *et al.*, 1996), the vast majority of the strains that have been identified are plant pathogens (Saddler & Bradbury, 2005). Collectively, they cause disease in a wide variety of flowering plant species including economically important crops such as brassicas, cereals, fruit trees, cassava, cotton and ornamentals (Leyns *et al.*, 1984; Hayward, 1993). However individually, they typically cause disease in a limited range of plants often within the same plant family (Saddler & Bradbury, 2005). The disease symptoms caused by xanthomonads are quite diverse and include necrosis, chlorosis, wilting, stunting, rots and cankers (Rudolph, 1993). Inside a host plant, many xanthomonads multiply locally in the intercellular spaces of the parenchyma tissues of leaves, stems and fruits, while others colonize the xylem vessels of the vascular system and spread systemically within the plant host (Rudolph, 1993; Büttner & Bonas, 2010; Ryan *et al.*, 2011). Overall, this diverse phytopathogenic specialization has strongly determined the classification of *Xanthomonas* bacteria leading to the establishment of over 140 pathovars within the genus (Vauterin *et al.*, 2000).

Early on, it was common practice among plant pathologists to define new bacterial species when pathogenic strains were isolated from a new plant host and numerous *Xanthomonas* species resulted from this so called 'new host-new species' approach (Starr, 1981). Since most of *Xanthomonas* species could only be distinguished by their host range, they were later merged in the single taxon *X. campestris* (Dye & Lelliott, 1974) and, subsequently, renamed as pathovars of *X. campestris* with the introduction of the descriptor pathovar in the nomenclature of phytopathogenic bacteria (Young *et al.*, 1978; Dye *et al.*, 1980).

The descriptor 'pathovar' was originally introduced to preserve information regarding distinctive pathogenicity of former bacterial species as a provisional solution until further research would provide better taxonomic resolution of those groups (Dye *et al.*, 1980; Vinatzer & Bull, 2009). However, the term pathovar remains a descriptor of common use to discriminate plant pathogenic bacteria below species level, based on differences in host range and/or disease symptoms induced on the same plant species (Dye *et al.*, 1980; Young *et al.*, 1991; Bull *et al.*, 2008). The

taxonomy of plant pathogenic bacteria has been regularly revised by the International Society for Plant Pathology, and valid pathovar names have been updated and published (Bull *et al.*, 2010a; Bull *et al.*, 2012). Additionally, the descriptor 'race' is also commonly used to distinguish groups of strains below species level, which are pathogenic to different sets of cultivars or other germplasm within one or more host species (Young *et al.*, 1991; Beattie, 2006; Vinatzer & Bull, 2009).

During the last decades, extensive examination of phenotypic and genotypic traits has improved the classification scheme of *Xanthomonas* bacteria to better represent their diversity and phylogenetic relationships (Gabriel *et al.*, 1989; Stead, 1989; Van Den Mooter & Swings, 1990; Palleroni *et al.*, 1993; Yang *et al.*, 1993; Vauterin *et al.*, 1995; Rademaker *et al.*, 2005; Saddler & Bradbury, 2005; Young *et al.*, 2008; Parkinson *et al.*, 2009; Rodriguez *et al.*, 2012). The most recent comprehensive re-classification of the genus was proposed by Vauterin *et al.* (1995) based on DNA homology. In this study, the genus was divided into 20 species of which, four species corresponded to previously defined species and 16 new species were composed of one or more former pathovars of *X. campestris* with few pathovars falling into more than one species (Vauterin *et al.*, 1995; Vauterin *et al.*, 2000). Debate followed the re-classification proposed by Vauterin *et al.* (1995) (Schaad *et al.*, 2000; Vauterin *et al.*, 2000), taxonomic refinements have been proposed and a few new species and subspecies have been described (Trebaol *et al.*, 2000; Jones *et al.*, 2004; Schaad *et al.*, 2005; Rademaker *et al.*, 2006; Schaad *et al.*, 2006; Young *et al.*, 2010).

Currently, the *Xanthomonas* genus comprises 28 validly described species (List of Prokaryotic names with Standing in Nomenclature; http://www.bacterio.net) and 72 pathovars defined within the species *X. axonopodis, X. arboricola, X. campestris, X. dyei, X. oryzae, X. translucens* and *X. vasicola* (Bull *et al.*, 2010a; Bull *et al.*, 2012) (Appendix 1). Additionally, approximatly 70 former pathovars of *X. campestris* remain with uncertain taxonomic identity and are classified as *X. campestris sensu lato* (Bull *et al.*, 2010a).

As debate continues on the *Xanthomonas* taxonomic framework, different designations for the same pathogen occur in the current literature as, for example, the causal agent of bacterial spot of tomato and pepper, *X. euvesicatoria* (Jones *et al.*, 2004), which is also commonly found in the literature designated as *X. campestris*

pv. *vesicatoria* (Thieme *et al.*, 2005; Büttner & Bonas, 2010). Although it is generally accepted that this pathogen does not belong to the species *X. campestris* (Vauterin *et al.*, 1995; Jones *et al.*, 2004), some authors have opted to continue using the classical nomenclature (Büttner & Bonas, 2010).

## 1.2 Pathogenic variants of *Xanthomonas campestris*

The species *X. campestris* (*sensu stricto*) has been described as including six pathovars that cause disease in crucifers (*i.e.* plants of the *Brassicaceae* family) according to the last emendation of the species based on a DNA-DNA hybridization study (Vauterin *et al.*, 1995). These pathovars are: *aberrans* (Knösel, 1961a; Dye *et al.*, 1980), *armoraciae* (McCulloch, 1929a; Dye *et al.*, 1980), *barbareae* (Burkholder, 1941; Dye *et al.*, 1980), *campestris* (Pammel, 1895; Dye *et al.*, 1980), *incanae* (Kendrick & Baker, 1942; Dye *et al.*, 1980), and *raphani* (White, 1930; Dye *et al.*, 1980) (Table 1). Additionally, *X. campestris* pv. *plantaginis* (Thornberry & Anderson, 1937; Dye *et al.*, 1980) has been described as a leaf spotting pathogen of ribgrass (family *Plantaginaceae*) and has also been added to the current emended description of *X. campestris* (Palleroni *et al.*, 1993; Vauterin *et al.*, 1995; Bull *et al.*, 2010a). However, the affiliation of this pathovar remains questionable (Saddler & Bradbury, 2005).

Four *X. campestris* pathovars (*aberrans*, *armoraciae*, *barbareae* and *raphani*) were originally described to cause leaf spot diseases, affecting localized areas of the parenchyma tissue without colonizing the vascular system of the host (McCulloch, 1929a; White, 1930; Knösel, 1961a; Bradbury, 1986), and two pathovars (*campestris* and *incanae*) have been described to cause vascular diseases by colonizing the vascular system of the plant host (Kendrick & Baker, 1942; Bradbury, 1986; Alvarez, 2000) (Table 1). However, the pathovar nomenclature within the species has not been clearly defined and several strains originally assigned to some of these pathovars (including pathotype strains) have been referred to as misidentified or non-pathogenic (Vauterin *et al.*, 1995; Schaad *et al.*, 2000; Vicente *et al.*, 2006; Fargier & Manceau, 2007; Vicente & Holub, 2013).

Fargier & Manceau (2007) suggested that the species *X. campestris* could be restricted to three pathovars namely, *campestris*, *incanae* and *raphani*, based on the distinct disease phenotypes caused by different strains in *Brassicaceae* hosts. Among the remaining pathovars, the few available strains assigned to pv. *aberrans* (including the pathotype strain) have been included in pv. *campestris* (races 5 and 8) because they were found to cause vascular infections contrarily to the original description of the pv. *aberrans* (Vicente *et al.*, 2001; Fargier & Manceau, 2007). The leaf spotting pv. *barbarea*, originally described as pathogenic on *Barbarea vulgaris* (a crucifer weed) (Burkholder, 1941), is under-represented in culture collections and the only two strains found available (including the pathotype strain) have been reported as non-pathogenic (Fargier & Manceau, 2007). And finally, the pathovars *armoraciae* and *raphani* have been considered synonymous by some authors (Black & Machmud, 1983; Alvarez *et al.*, 1994; Zhao *et al.*, 2000a; Pernezny *et al.*, 2003) whereas other authors have considered that both pathovars should stand based on their different host range and the disease symptoms they cause (Tamura *et al.*, 1994; Vicente *et al.*, 2006).

**Table 1**. Summary of *Xanthomonas campestris* pathovars (*sensu stricto*), associated disease symptoms, respective natural hosts and geographical distribution

| Pathovar [a] | Disease: symptoms (reference) | Natural hosts (reference) | Geographical distribution (reference) | |
|---|---|---|---|---|
| ***Leaf spot diseases*** | | | | |
| ***aberrans*** ([1]) | Leaf spot: numerous small necrotic leaf spots without infection of the vascular system (**10**) | Crucifer:<br>*Brassica oleracea* var. *botrytis* (cauliflower) (**10**) | Germany (**10**)<br>South Africa (**3**) | |
| ***armoraciae*** ([2]) | Leaf spot: leaf spots enlarge and coalesce and are not limited by veins; vascular invasion beyond necrotic lesions not observed (**13**) | Crucifer:<br>*Armoracia rusticana* (horse-radish) (**2**, **4**, **7**, **13**) | India and probably Turkey **(3)**<br>Twain (**7**)<br>USA (**2**, **4**, **13**) | |
| ***barbareae*** (ATCC13460, CFBP5825, HRI8520, ICMP438; LMG547, NCPPB983) | Leaf spot: small leaf spots spreading to the stem and killing of leaves (**4**) | Crucifer weed:<br>*Barbarea vulgaris* (winter cress) (**4**) | USA (**4**) | |
| ***raphani*** (ATCC49079, CFBP5827, ICMP1404, LMG860, NCPPB1946 [3]) | Leaf spot: leaf spots may enlarge and coalesce and are not limited by veins; vascular invasion beyond necrotic lesions not observed; black, sunken and elongated lesions in stems and petioles (**23**); black and sunken spots on harvested radish hypocotyls and roots (**18**) | Crucifers and tomato:<br>*B. oleracea* (cabbage, cauliflower) (**5**, **19**, **22**, **25**)<br>*B. rapa* (turnip, Chinese cabbage, spinach mustard) (**19**, **22**, **23**)<br>*Raphanus sativus* (radish) (**6**, **12**, **18**, **19**, **22**, **23**)<br>*Solanum lycopersicum* (tomato) (**3**, **11**, **15**, **16**, **22**) | Brazil (**16**)<br>Canada (**11**, **22**)<br>East Asia (**22**)<br>France (**22**)<br>Italy (**12**) | Japan (**19**)<br>Portugal (**5**)<br>Russia (**15**)<br>Taiwan (**25**)<br>Turkey and India (**3**)<br>USA (**5, 6**, **18**, **22**, **23**) |
| ***Vascular diseases*** | | | | |
| ***campestris*** (ATCC33913, CFBP5251, HRI5212, ICMP13, LMG568, NCPPB528) | Black rot: blackening of the vascular tissue; foliar V-shaped chlorotic/necrotic lesions; plant wilt, stunting and misshaping (**1**, **3**, **24**); rotting of roots (**14**) | Several crucifers:<br>*Brassica* spp. (**3**, **6**, **21, 24**)<br>*R. sativus* (radish) (**3**, **24**)<br>*Capsella bursa-pastoris* and other cruciferous weeds including *Arabidopsis thaliana* (**3**, **8**, **17, 20**, **21**, **24**) | Worldwide (**1, 3**, **6**, **21**, **24**) | |
| ***incanae*** (ATCC13462, CFBP2527, HRI6377, ICMP574, LMG7490, NCPPB937) | Bacterial blight: black lesions along the main stem; blackening of the vascular tissue; yellowing of leaves; plants may wilt and collapse or show stunting growth (**9, 3**) | Ornamental crucifers:<br>*Matthiola incana* (stock) (**6**, **9**)<br>*Matthiola* sp. (stock) (**3**)<br>*Cheiranthus cheiri* (wallflower) (**3**) | Australia and South Africa (**3**)<br>France (**6**)<br>USA (**9**) | |

References: **1**, Alvarez (2000); **2**, Anderson & Thornberry (1938); **3**, Bradbury (1986); **4**, Burkholder (1941); **5**, Cruz *et al.* (2015); **6**, Fargier & Manceau (2007); **7**, Hseu *et al.* (2009); **8**, Ignatov *et al.* (2007); **9**, Kendrick & Baker (1942); **10**, Knösel (1961a; 1961b); **11**, Kuflu & Cuppels (1997); **12**, Loreti *et al.* (2011); **13**, McCulloch (1929a; 1929b); **14**, Pammel (1895); **15**, Punina *et al.* (2009); **16**, Robbs (1961); **17**, Schaad & Dianese (1981); **18**, Segall & Smoot (1962); **19**, Tamura *et al.* (1994); **20**, Tsuji & Somerville (1992); **21**, Vicente *et al.* (2001); **22**, Vicente *et al.* (2006); **23**, White (1930); **24**, Williams (1980); **25**, Wu *et al.* (2007).

[a] Pathotype strains are indicated within brackets according to Bull *et al.* (2010a) except for strains that have been reported as misidentified or assigned to other pathovar as follows: [1] The pathotype strain of *X. campestris* pv. *aberrans* (HRI3880, NCPPB2986, CFBP6865) has been assigned to *X. campestris* pv. *campestris* race 5 (Vicente *et al.*, 2001; Fargier & Manceau, 2007); [2] The pathotype strain of *X. campestris* pv. *armoraciae* (HRI6375, NCPPB347, CFBP3838) is misidentified and it is a vascular pathogen of candytuft (Vicente *et al.*, 2006; Fargier & Manceau, 2007); [3]The pathotype strain of *X. campestris* pv. *raphani* has been reported as misidentified by some authors (Vicente *et al.*, 2001; 2006) whereas other authors have described it as an authentic strain (Benoit *et al.*, 2000; Fargier & Manceau, 2007; Loreti *et al.*, 2011).

Few *X. campestris* strains have also been described to cause vascular diseases in the ornamental crucifers wallflower and candytuft (Vicente *et al.*, 2001; Fargier & Manceau, 2007). Vicente *et al.* (2001, 2006) proposed that these strains should be assigned to new pathovars, whereas Fargier & Manceau (2007) recommended that strains pathogenic to wallflower should be assigned to pv. *incanae* (as described by Bradbury, 1986) and a strain found pathogenic to candytuft should be assigned to pv. *campestris*. This latter strain has been described as the pathotype strain of *X. campestris* pv. *armoraciae* (NCPPB347, CFBP3838, HRI6375) (Dye *et al.*, 1980; Bull *et al.*, 2010a) but it is misidentified because it does not cause leaf spot disease in horseradish as described for this pathovar, and instead, it causes a vascular disease in candytuft and radish (Vicente *et al.*, 2001; Vicente *et al.*, 2006; Fargier & Manceau, 2007).

Within the diverse range of *X. campestris* pathogenic variants, the pathovars *raphani* and *campestris* stand as contrasting examples that infect common plant species (brassicas and radish), but have distinct host tissue specificities and cause distinct disease symptoms (leaf spot and vascular symptoms, respectively) as shown in Figure 1.

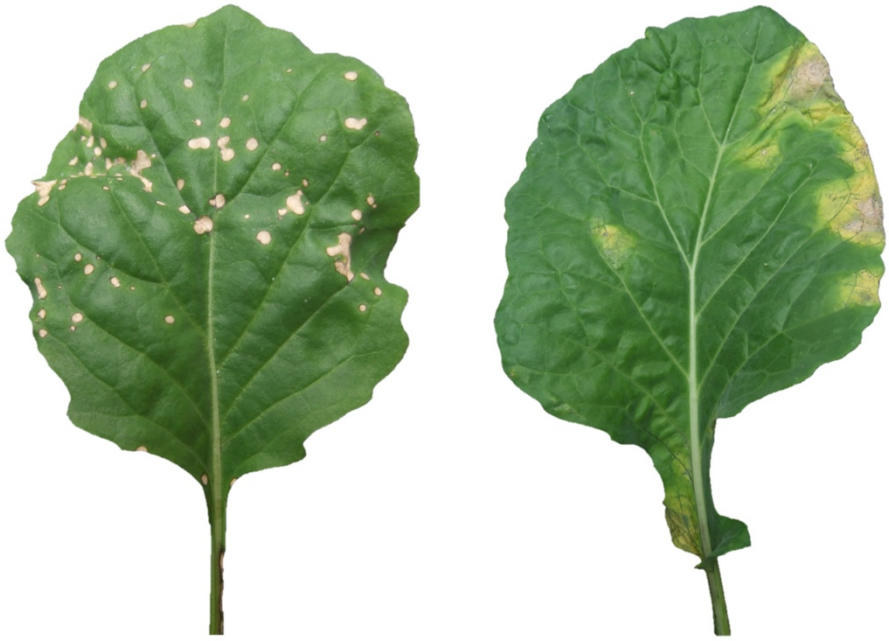

**Figure 1**. Typical disease symptoms caused by *Xanthomonas campestris* pv. *raphani* (leaf spots; left) and *X. campestris* pv. *campestris* (black veins and V-shaped chlorotic/necrotic lesions; right) in *Brassica oleracea* var. *sabauda* cv. Wirosa (Savoy cabbage).

### 1.2.1 *Xanthomonas campestris* pv. *raphani*

*Xcr* was first described by White (1930) as the causal pathogen of a leaf spot disease in radish and turnip occurring in Indiana (USA) in 1928. The different names attributed to this pathogen since its first description, are: *Bacterium vesicatorium* var. *raphani* (White, 1930), *X. vesicatoria* var. *raphani* and *X. campestris* pv. *raphani* (Young *et al.*, 1978; Dye *et al.*, 1980).

*Xcr* was originally designated *B. vesicatorium* var. *raphani* because it resembled *B. vesicatorium* (currently designated *X. euvesicatoria or X. campestris* pv. *vesicatoria*) in the symptoms produced in tomato plants, but differed from the latter by being pathogenic to a wider host range that included not only tomato and pepper (as for *B. vesicatorium*), but also brassicas and tobacco (White, 1930). *Xcr* was also originally described to resemble *X. campestris* pv. *armoraciae* McCulloch (1929a), but it was distinguished from this pathovar by having a wider host range that included several brassicas, tomato, pepper and tobacco, but not horseradish, whereas the pv. *armoraciae* had been described pathogenic to horseradish and only weakly pathogenic in cauliflower and cabbage (McCulloch, 1929a). Furthermore,

sunken lesions in petioles and stems were only mentioned in the original description of pv. *raphani* (White, 1930).

*Xcr* invades the plant tissue through stomata or wounds and colonizes the parenchyma tissues of the host without colonizing the vascular system (White, 1930; Tamura *et al.*, 1994). Typical disease symptoms caused by *Xcr* include well-defined necrotic spots in leaves that may enlarge and coalesce (Figure 1) and dark sunken lesions in petioles and stems that can progress deeply to cause breakage of petioles and killing of young plants (White, 1930; Tamura *et al.*, 1994; Vicente *et al.*, 2006). Post-harvest symptoms characterized by black sunken spots in radish hypocotyls and roots with little internal discoloration have also been associated to *Xcr* (Segall, 1961; Segall & Smoot, 1962), and were first reported in Florida when the packaging and shipping of radishes in plastic bags had started (Thompson & Halsey, 1954b; 1954a; Thompson & Decker, 1955; Segall, 1961; Segall & Smoot, 1962). White (1930) showed that *Xcr* is seed transmitted. Evidence for possible dissemination of *Xcr* by vectors was reported by Benoit *et al.* (2000) using tardigrades that could shed and transmit viable pathogenic *Xcr* bacteria to radish plants.

The presence of *Xcr* has been detected in several countries around the globe including the USA, Brazil, Canada, India, Japan, Taiwan, Russia, Turkey, France and recently in Italy and Portugal (Table 1). *Xcr* has been isolated from field and glasshouse grown plants as well as seed lots (Vicente *et al.*, 2006; Punina *et al.*, 2009; Loreti *et al.*, 2011; Cruz *et al.*, 2015), but the impact of this pathovar in crop production is not well documented. Natural hosts of *Xcr* include plants from the *Brassicaceae* family (cabbage, cauliflower, turnip, Chinese cabbage, spinach mustard and radish) and *Solanaceae* family (tomato) (Table 1). Additionally, a wide range of susceptible plants from different families have been identified by artificial inoculation, as follows: *Brassicaceae* (kale, broccoli, Ethiopian mustard, black mustard, Brussels sprouts, oilseed rape, ornamentals candytuft and wallflower, and the plant model *A. thaliana*), *Solanaceae* (pepper, physalis and tobacco), *Cucurbitaceae* (cucumber and pumpkin) and *Fabaceae* (soybean) (White, 1930; Segall & Smoot, 1962; Bradbury, 1986; Parker *et al.*, 1993; Tamura *et al.*, 1994; Vicente *et al.*, 2006; Fargier & Manceau, 2007; Wu *et al.*, 2007; Punina *et al.*, 2009; Loreti *et al.*, 2011).

The pathotype strain of *Xcr* is available in several culture collections with the following accession numbers, ATCC49079, CFBP5827, ICMP1404, LMG860 and NCPPB1946 (Bull *et al.*, 2010a). Vicente *et al.* (2006) reported that the pathotype strain of *Xcr* is mislabelled and should be assigned to *Xcc*, but authentic clones of this strain obtained from different collections have been described in other studies (Benoit *et al.*, 2000; Fargier & Manceau, 2007; Loreti *et al.*, 2011).

### 1.2.2 *Xanthomonas campestris* pv. *campestris*

*Xcc* is the causal agent of black rot, one of the most important diseases of crucifers worldwide (Williams, 1980; Schaad & Alvarez, 1993; Alvarez, 2000). Early reports of the disease date from 1889 (Alvarez, 2000) although the causal pathogen was only described in 1895 following a severe root rot in *Brassica campestris* (synonym of *B. rapa*) occurring in Iowa (USA) (Pammel, 1895). Since its first description, its name has changed as follows: *Bacillus campestris*, *Pseudomonas campestris*, *Bacterium campestris*, *Phytomonas campestris*, *X. campestris* and *X. campestris* pv. *campestris* (Alvarez, 2000).

*Xcc* typically enters the plant host through hydathodes at the leaf margins or wounds, although invasion via stomata and natural openings in roots may also occur (Cook *et al.*, 1952; Alvarez, 2000). Inside the plant, bacteria colonize primarily the xylem vessels and move systemically throughout the plant (Williams, 1980; Bretschneider *et al.*, 1989; Alvarez, 2000). The typical symptoms caused by *Xcc* include darkening of veins and chlorotic/necrotic V-shaped lesions starting at the margins of the leaves (Alvarez, 2000) (Figure 1). As the disease progresses, plants may wilt or become stunted and secondary infections by soft-rotting bacteria such as *Pectobacterium carotovorum* (syn. *Erwinia carotovora*) and *Pseudomonas marginalis*, often occur leading to the rotting of plants (Williams, 1980). Although symptoms in roots are rarely described in the literature, *Xcc* may also cause darkening of vascular tissues in roots followed by root rotting as described by Pammel (1895) in swede and turnip.

*Xcc* 'is found wherever crucifers are grown' (Schaad & Alvarez, 1993). It has been identified in all common cultivated crucifers such as, *Brassica oleracea* (cabbage, kale, collard, broccoli, cauliflower, kohlrabi, Brussels sprouts), *B. rapa*

(turnip, Chinese cabbage), *B. juncea* (mustards), *B. napus* (oilseed rape, swede) and *Raphanus sativus* (radish) (Bradbury, 1986); as well as numerous crucifer weeds (Schaad & Dianese, 1981; Bradbury, 1986; Ignatov *et al.*, 2007) including *A. thaliana* (Tsuji & Somerville, 1992).

Seeds, crop residues and weeds are important sources of inoculum for black rot (Schaad & Alvarez, 1993) and temperatures above 25 °C and humid conditions favour the development of the disease (Williams, 1980; Alvarez, 2000).

## 1.3 Pathogenicity factors of *Xanthomonas*

*Xanthomonas* bacteria depend on an array of pathogenicity factors to successfully colonize plant hosts. Known pathogenicity factors include lipopolysaccharides that are main components of the bacterial outer membrane, the extracellular polysaccharide xanthan, extracellular degradative enzymes, adhesins anchored in the bacterial outer membrane and the type III secretion system (T3SS) (Büttner & Bonas, 2010; Ryan *et al.*, 2011).

The T3SS is essential for pathogenicity of *Xanthomonas* spp. including *Xcr* and *Xcc*, as well as other bacterial pathogens (Arlat *et al.*, 1991; Kamoun *et al.*, 1992; Büttner & Bonas, 2010). The T3SS is a specialized syringe-like apparatus that bacteria utilize to translocate effector proteins (T3 effectors) into the host cells (Büttner & He, 2009). Mutation of genes encoding structural components of this system (*hrp* or hypersensitive and pathogenicity genes), results in complete loss or drastic reduction of pathogenicity indicating that the translocation of effector proteins is essential for disease development; yet mutation of individual T3 effector genes often does not significantly affect pathogenicity possibly due to functional redundancy among co-secreted effectors (Alfano & Collmer, 1997; Büttner & Bonas, 2010; White *et al*., 2009). Collectively, T3 effectors are known to manipulate plant cellular processes for the benefit of the pathogen, including suppression of host defense responses, but may also betray the pathogen and trigger plant defense responses (Alfano & Collmer, 2004; Grant *et al.*, 2006; Macho, 2016).

The identification of T3 effectors or effector candidates of *Xanthomonas* bacteria has greatly advanced with the availability of genome sequences of several

strains (White *et al.*, 2009; Ryan *et al.*, 2011). An extensive list of known or candidate T3 effectors that have been identified in *Xanthomonas* spp., is available in the *Xanthomonas* Resource database (http://www.xanthomonas.org/t3e.html; White *et al.*, 2009). At the beginning of this study, complete genome sequences of one *Xcr* strain (Bogdanove *et al.*, 2011) and three strains of *Xcc* (da Silva *et al.*, 2002; Qian *et al.*, 2005; Vorhölter *et al.*, 2008) were publically available. A total of eight T3 effector genes have been identified in the *Xcr* genome whereas at least 20 T3 effector genes have been identified in the three complete genomes of *Xcc* (http://www.xanthomonas.org/t3e.html).

Among *X. campestris* pathovars, *Xcc* has been the most studied and several pathogenicity determinants have been identified in strains of this pathovar (Alvarez, 2000; Qian *et al.*, 2005; Vicente & Holub, 2013). The pathogenicity determinants of *Xcr* are mostly unknown and the molecular basis of the distinct modes of pathogenesis of these pathovars remains elusive.

## 1.4 Plant immunity

Major progress has been made over the recent decades of research on identifying key molecular players involved in plant-pathogen interactions (Staskawicz, 2001; Dangl & Jones, 2001; Chisholm *et al*., 2006; Mansfield, 2009; Dodds & Rathjen, 2010). During plant colonization, successful pathogens must overcome plant constitutive defences such as waxy cuticle layers, cell walls and pre-existing antimicrobial compounds, as well as plant inducible defence responses (Agrios, 2005; Chisholm *et al*., 2006). Various pathogens secrete effector molecules to the extracellular and/or intracellular spaces of the plant host to enable parasitism (Hogenhout *et al.*, 2009; Win *et al.*, 2012). In turn, plants rely on immune receptor proteins, at the cell surface and inside cells, for perception of pathogen invasion and activation of effective defence responses (Dangl *et al.*, 2013).

Plant immune receptors localized at the cell surface perceive evolutionary conserved pathogen or microbial molecules, pathogen effectors and plant molecules released during pathogen invasion (Thomma *et al.*, 2011; Monaghan & Zipfel, 2012; Win *et al.*, 2012). These receptors include kinases that have an extracellular leucine

rich repeat or lysine domain, or similar proteins that lack the kinase domain, and are generally designated pattern recognition receptors (PRRs) (Dangl *et al.*, 2013; Zipfel, 2014). Examples of well-characterized PRRs include FLS2, EFR and CERK1 that recognize the widely conserved bacterial flagellin, bacterial elongation factor Tu and fungal cell wall component chitin, respectively (Zipfel, 2014). Other example is the rice receptor Xa21 that recognizes a recently identified protein of *X. oryzae* pv. *oryzae* (RaxX) and confers robust resistance to most strains of this pathogen (Zipfel, 2014; Pruitt *et al.*, 2015).

Inside plant cells, specific immune receptors enable recognition of specific effectors that pathogens translocate into the host cells to suppress PRR-dependant defence responses as well as to gain access to nutrients and facilitate their survival and dispersal (Dangl *et al.*, 2013). These receptors are typically highly variable proteins that have a central nucleotide-binding domain (NB) and a C-terminal leucine rich repeat domain (LRR), either localized at the plasma membrane or in the cytosol or shuttling between the cytosol and nucleus (Bonardi *et al.*, 2012; Dangl *et al.*, 2013). NB-LRR proteins can recognize pathogen effectors either directly by physical contact or indirectly through the interaction between pathogen effectors and plant accessory proteins that are part of the NB-LRR protein complex (Dodds & Rathjen, 2010; Ntoukakis *et al.*, 2014). For example, the flax M protein directly interacts with the effector AvrM of the flax rust fungus (Catanzariti *et al.*, 2010), whereas for example, the *A. thaliana* NB-LRRs RPM1 and RPS2 recognize the *P. syringae* effectors AvrRpm1/AvrB and AvrRpt2 respectively through modification of the plant protein RIN4 (Dodds & Rathjen, 2010), and the tomato Pto kinase interacts with the *P. syringae* effectors AvrPto and AvrPtoB and confers the recognition function to the tomato NB-LRR Prf (Ntoukakis *et al.*, 2014). Recognition of pathogen effectors via NB-LRR proteins leads to effective and rapid defence responses that halt pathogen proliferation, which are often, but not always, associated to localized plant cell death (or hypersensitive response) (Dodds & Rathjen, 2010; Coll *et al.*, 2011; Dangl *et al.*, 2013).

In addition to PRRs and NB-LRRs, other disease resistance mechanisms involve for example genes that allow recognition of transcription activator like (TAL) effectors which are DNA binding proteins translocated into the host cells by *Xanthomonas* and *Ralstonia* bacteria. These effectors activate plant gene expression

to enhance virulence, but may also be recognized by the plant host through specific genes that have TAL effector binding sites in their promoters. The transcriptional activation of those plant genes by TAL effectors induces hypersensitive host cell death and limits pathogen proliferation (Dangl *et al.*, 2013).

## 1.5 Predicted gene-for-gene models to explain interactions between *Xanthomonas campestris* pathovars *raphani* and *campestris*, and *Brassicaceae* hosts

The gene-for-gene concept was introduced by Harold Flor to explain the genetic inheritance of both, monogenic disease resistance in cultivars of flax and virulence in pathogenic variants of the flax rust fungus (Flor, 1971). This concept states that for each gene that confers resistance (*R* gene) in the host, there is a corresponding gene in the pathogen that confers avirulence (avirulence gene), and these corresponding gene pairs are necessary for disease resistance to occur. The simplest form of this concept has been supported by the identification of several plant *R* genes whose products mediate recognition of specific pathogen effectors (Dangl & Jones, 2001; Chisholm *et al*., 2006).

Phenotypic variation has been identified in interactions between *Xcr* and *Xcc* strains and *Brassicaceae* plant lines. Races of both pathogens have been described based on compatible and incompatible interactions between several strains and some common plant lines as summarized in Table 2. Three *Xcr* races have been described thus far using plant lines of five *Brassica* species (*B. oleracea*, *B. carinata*, *B. juncea*, *B. napus* and *B. rapa*) and radish (*Raphanus sativus*) (Vicente *et al.*, 2006). Within *Xcc*, nine races have been defined using *Brassica* spp. lines, some of which also discriminate *Xcr* races (Vicente *et al.*, 2001; Fargier & Manceau, 2007; Jensen *et al.*, 2010; Vicente & Holub, 2013).

Gene-for-gene models have been proposed to describe the differential interactions between *Brassicaceae* plant lines and races of *Xcr* and *Xcc* as summarized in Table 2 (Vicente *et al.*, 2006; Fargier & Manceau, 2007; Vicente & Holub, 2013). These models assumed the minimum number of gene pairs to explain those interactions. Thus far, none of the resistance or avirulence genes predicted in

these models have been confirmed. However, there is genetic evidence to support the existence of three predicted resistance genes to *Xcc* races, namely *R1c*, *R3c* and *R4c*. A single genetic locus conferring dominant resistance to *Xcc* race 4 has been mapped in *B. napus* and may correspond to *R4c*. The existence of a single locus conferring dominant resistance to *Xcc* races 1 and 4 in *B. carinata* (that may correspond to *R1c*) and a single locus conferring dominant resistance to *Xcc* race 3 in *B. oleracea* (that may correspond to *R3c*), have been confirmed by inheritance of resistance studies (Vicente *et al.*, 2002). Furthermore, genes that confer avirulence to *Xcc* strains in *Brassica* spp. lines have been identified (Castaneda *et al.*, 2005; He *et al.*, 2007) and it is possible that the genes *xopAH* and *xopE* correspond to the avirulence genes *A1c* and *A4c* predicted in the *Xcc* model (Vicente & Holub, 2013). Regarding *Xcr*, no genetic studies have been carried out to confirm predictions of resistance genes and avirulence genes in the model.

Within the small group of *Xcr* strains race-typed so far, most strains of races 1 and 3 were found in *B. oleracea* whereas strains of race 2 were mostly found in *B. rapa* (Vicente *et al*., 2006). In *Xcc*, a much higher number of strains have been race-typed and races 1 and 4 are frequently found in *B. oleracea* (Vicente *et al.*, 2001; Taylor *et al.*, 2002; Jensen *et al.*, 2010; Mulema *et al.*, 2012; Vicente & Holub, 2013) whereas race 6 is common in *B. rapa* (Vicente *et al.*, 2001; Lema *et al.*, 2012; Vicente & Holub, 2013) and has the widest host range among all *Xcc* races (Taylor *et al.*, 2002). Other *Xcc* races are less commonly reported (Vicente & Holub, 2013).

**Table 2**. Postulated gene-for-gene models to explain the interactions between *Brassicaceae* plant lines and races of *Xanthomonas campestris* pathovars *raphani* and *campestris* [a]

| | Resistance genes (*R*) | | | | | | | | Races and avirulence genes (*A*) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | *Xcr* | | | *Xcc* | | | | | | | | | |
| | | | | | | | | | **1** | **2** | **3** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** |
| | | | | | | | | | *A1r* | *A2r* | . | *A1c* | . | *A1c* | *A1c* | . | . | . | *A1c* | *A1c* |
| | | | | | | | | | . | . | . | . | *A2c* | . | . | . | . | . | *A2c* | . |
| | | | | | | | | | . | . | . | . | *A3c* | *A3c* | . | *A3c* | . | . | *A3c* | *A3c* |
| | | | | | | | | | . | . | . | . | . | . | *A4c* | . | . | . | . | *A4c* |
| **Plant cultivar/line (species)** | *Xcr* | | | *Xcc* | | | | | . | . | . | . | . | . | . | *A5c*? | . | *A5c* | . | . |
| Wirosa (*Brassica oleracea*)[b] | . | . | . | . | . | . | . | . | + | + | + | + | (+)/+ | + | + | + | + | + | + | + |
| French Breakfast (*Raphanus sativus*)[b] | . | . | . | . | . | . | . | . | + | + | + | . | . | . | . | . | . | . | . | . |
| PIC1 (*B. carinata*)[c] | . | . | . | *R1c* | . | . | . | . | + | + | + | − | (+) | − | − | + | + | + | − | − |
| FBLM2 (*B. juncea*)[c] | *R1r* | . | . | *R1c* | . | . | . | *R5c* | − | + | + | − | + | − | − | (+) | + | − | − | − |
| Just Right Hybrid Turnip (*B. rapa*)[b] | *R1r* | . | . | . | . | . | *R4c* | . | − | + | + | + | + | + | − | + | + | + | + | − |
| COB60 (*B. napus*)[c] | *R1r* | . | . | . | . | . | *R4c* | . | − | + | + | + | (+) | + | − | + | + | + | . | . |
| Mino Early (*R. sativus*)[b] | . | *R2r* | . | . | . | . | . | . | + | − | + | . | . | . | . | . | . | . | . | . |
| Miracle (*B. oleracea*)[b] | . | *R2r* | . | . | . | *R3c* | . | . | + | − | + | + | −/(+) | −/(+) | + | − | + | + | − | − |
| SxD1 (*B. oleracea*)[c] | . | . | . | . | . | *R3c* | . | . | . | . | . | + | −/(+) | − | + | − | (+)/+ | + | . | . |
| Seven Top Turnip (*B. rapa*)[b] | . | . | . | . | *R2c* | . | *R4c* | . | . | . | . | + | − | v | v | v | + | + | − | − |

Symbols and abbreviations: +, susceptibility; −, resistance; (+), weakly pathogenic; v, variable; *Xcc*, *Xanthomonas campestris* pv. *campestris*; *Xcr*, *X. campestris* pv. *raphani*.

[a] Adapted from Vicente *et al.* (2006) and Vicente & Holub (2013).

[b] Commercial cultivars.

[c] Non-commercial lines: COB60 is a double haploid line obtained by microspore culture from a selection of *B. napus* cv. Cobra line 14R that was resistant to *Xcc* race 4; PIC1 is a doubled haploid obtained by microspore culture from a plant of a selection of *B. carinata* accession PI199947; FBLM2 is a doubled haploid obtained by microspore culture from a plant of *B. juncea* cv. Florida Broad Leaf Mustard; SxD1 is a doubled haploid obtained by microspore culture from a plant derived from a cross between *B. oleracea* cv. Böhmerwaldkohl and a rapid cycling line (J. Vicente, pers. comm.).

## 1.6 The plant model *Arabidopsis thaliana*

*Arabidopsis thaliana* is a wild flowering plant that belongs to the *Brassicaceae* family and is a close relative of several cultivated plants such as brassicas and radish. Its small size, short life cycle, prolific seed production, self-compatibility, and relatively small genome (five chromosomes, 125 Mb) have contributed to the development of *A. thaliana* as an experimental model in plant biology research (Meyerowitz & Somerville, 1994; https://www.arabidopsis.org).

Natural *A. thaliana* accessions have been collected from diverse geographic locations around the globe and have enabled researchers to study the molecular basis of natural phenotypic variation of different traits such as flowering time and disease resistance (Alonso-Blanco & Koornneef, 2000; Weigel, 2012). Columbia was the first accession having the complete genome sequenced (AGI, 2000) and the genomes of several additional accessions are being sequenced and published in a joint effort of the research community that incorporate the 1001 Genomes Project (http://1001genomes.org; Weigel & Mott, 2009; Cao *et al.*, 2011; Gan *et al.*, 2011). Other useful genetic resources are, for example, recombinant inbred mapping populations including populations derived from crosses of two parents (Alonso-Blanco & Koornneef, 2000) and inter-crossing of multiple parents (Kover *et al.*, 2009), as well as a large collection of T-DNA (transfer DNA) insertion mutants that encompass potential loss of function of most genes distributed across the *A. thaliana* genome (McElver *et al.*, 2001; Alonso *et al.*, 2003; http://signal.salk.edu/cgi-bin/tdnaexpress). In addition, genetic transformation of *A. thaliana* can be easily performed (Bent, 2006) which is a major advantage for molecular genetic studies. The publically available TAIR database (The Arabidopsis Information Resource; https://www.arabidopsis.org/) provides a useful compilation of information and tools that supports research using *A. thaliana*. Seed samples of biological materials are available from seed stock centres such as ABRC (Arabidopsis Biological Resource Centre, USA; https://abrc.osu.edu) and NASC (Nottingham Arabidopsis Stock Centre, UK; http://arabidopsis.info).

## 1.7 Molecular basis of resistance in *Arabidopsis thaliana* to *Xanthomonas campestris*

*A. thaliana* has proved to be a useful model to study the mechanisms of disease resistance aided by the substantial development of genetic tools and resources by a multinational research community (Nishimura & Dangl, 2010). For example, strains of the bacterium *Pseudomonas syringae* (causing bacterial speck) and the oomycete *Hyaloperonospora arabidopsidis* (causing downy mildew) have been used extensively to investigate the molecular basis of *R*-gene mediated perception of pathogen effectors and downstream defence signalling (Grant *et al.*, 2006; Holub, 2008; Nishimura & Dangl, 2010; Xin & He, 2013; McDowell, 2014).

*Xcc* was among the earliest pathogens described to infect *A. thaliana* (Tsuji & Somerville, 1988; Simpson & Johnson, 1990). However, the understanding of the molecular basis of resistance to *Xcc* in this pathosystem has progressed slowly. Within the species *X. campestris*, other pathogenic variants have also been used to investigate responses in *A. thaliana* (Table 3), but research has mostly been pursued with *Xcc* (Buell, 2002; Meyer *et al.*, 2005).

**Table 3**. Examples of *Xanthomonas campestris* pathovars examined on *Arabidopsis thaliana* [a]

| ***Xanthomonas campestris*** **pathovars** | **Sample references** |
|---|---|
| *X. campestris* pv. *aberrans* | Parker *et al.* (1993) |
| *X. campestris* pv. *armoraciae* | Davis *et al.* (1991)<br>Hugouvieux *et al.* (1998) |
| *X. campestris* pv. *campestris* | Simpson & Johnson (1990)<br>Davis *et al.* (1991)<br>Parker *et al.* (1993)<br>Tsuji & Somerville (1988)<br>Tsuji *et al.* (1991)<br>Lummerzheim *et al.* (1993)<br>Hugouvieux *et al.* (1998)<br>Meyer *et al.* (2005)<br>Guy *et al.* (2013)<br>Huard-Chauveau *et al.* (2013) |
| *X. campestris* pv. *raphani* | Parker *et al.* (1993)<br>Davis *et al.* (1991) |

[a]Adapted from Buell (2002).

Initial screens of phenotypic variation among interactions between *Xcc* strains and *A. thaliana* accessions showed differential responses, and attempts were then made to genetically map the disease resistance loci (Simpson & Johnson, 1990; Daniels *et al.*, 1991; Tsuji *et al.*, 1991; Lummerzheim *et al.*, 1993; Buell & Somerville, 1997). For example, Tsuji *et al.* (1991) reported that a single dominant locus (designated *RXC1*) confers tolerance in the accession Columbia to a strain of *Xcc*, which was subsequently mapped to chromosome 2 by Buell & Somerville (1997). The latter authors also mapped three resistance loci using the same *Xcc* strain, including *RXC2* on chromosome 5 which confers dominant resistance by a single gene, and *RXC3* and *RXC4* on chromosomes 5 and 2 respectively, which function together in a digenic manner. Godard *et al.* (2000) screened mutants of *A. thaliana* Columbia and mapped a locus on chromosome 3 involved in resistance to a strain of *Xcc*.

Despite these early efforts, genes involved in the recognition of *Xcc* have only recently begun to be identified in *A. thaliana*. Huard-Chauveau *et al.* (2013) studied the genetic basis of resistance to the *Xcc* strain ATCC33913. In this study, a gene conferring resistant to *Xcc* was identified on chromosome 3 of the accession Columbia and encodes a kinase-like protein. In another recent study, two major effect loci conferring resistance to *Xcc* were identified on chromosome 5. In one locus, a gene encoding a protein of unknown function was reported to confer resistance to a *Xcc* strain of race 2 in the accession Columbia; in the second locus, the well-known disease resistance gene pair *RRS1*/*RPS4* was reported to confer resistance to a *Xcc* strain of race 6 in the accession Wassilewskija (Ws-0) (Debieu *et al.*, 2016).

Investigation of phenotypic variation in responses of *A. thaliana* accessions to other *X. campestris* pathovars has been conducted, using for example strains assigned to the leaf spotting pathovars *Xcr* and *X. campestris* pv. *armoraciae* (Davis *et al.*, 1991; Parker *et al.*, 1993). However, most strains were described as avirulent or producing weak symptoms and only the pathotype strain of *Xcr* (NCPPB1946) was found virulent in all *A. thaliana* accessions tested by Parker *et al.* (1993). In these studies, infiltration of leaves was used, which is an inoculation method that bypasses the natural entry of these pathogens via stomata, and this might have influenced the interactions observed. Furthermore, Hugouvieux *et al.* (1998)

reported that a *X. campestris* pv. *armoraciae* strain was capable of colonizing *A. thaliana* leaves via stomata in the first hours after inoculation, but did not report further development of disease symptoms nor characterized the interactions between this strain and different *A. thaliana* accessions. Although there is evidence that leaf spotting *X. campestris* strains can infect *A. thaliana* under laboratory conditions (Parker *et al.*, 1993; Hugouvieux *et al.*, 1998), research did not progress to explore the interactions between these pathogens and *A. thaliana*.

## 1.8 Aims and objectives

Characterization of interactions between *A. thaliana* and *Xcr* was a major aim of this thesis research. The specific objectives were to:

1. Characterize the natural phenotypic variation of resistance and susceptibility among accessions of *A. thaliana* to the three known races of *Xcr* and three important races of *Xcc* that have been described based on interactions with *Brassicaceae* hosts.
2. Determine the molecular basis of resistance in *A. thaliana* to at least one race of *Xcr*.

Two additional objectives focused on investigating pathogen characteristics including:

3. Molecular and pathogenic characterization of *X. campestris* strains associated with outbreaks of a severe leaf spot and blight disease of field-grown brassicas in Mauritius.
4. Identification of candidate determinants of phenotypic variation between *Xcr* and *Xcc* and races within both pathovars using comparative analysis of whole-genome sequencing data.

# CHAPTER 2. Phenotypic variation within *Arabidopsis thaliana* for resistance to *Xanthomonas campestris* pv. *raphani*

## 2.1 INTRODUCTION

The *Xanthomonas campestris* species comprises distinct pathogenic variants that infect *Brassicaceae* hosts, including *X. campestris* pv. *raphani* (*Xcr*) and *X. campestris* pv. *campestris* (*Xcc*). These two pathovars are generally adapted to different modes of infection and cause distinct disease symptoms. *Xcr* typically enters the host via stomata, colonizes the parenchyma tissues and causes leaf spots (White, 1930; Tamura *et al.*, 1994; Vicente *et al.*, 2006), whereas *Xcc* typically enters the host via hydathodes to colonize the xylem vessels and causes blackening of veins and extended chlorotic/necrotic V-shaped lesions in leaves (Williams, 1980; Bretschneider *et al.*, 1989; Alvarez, 2000).

Among *X. campestris* bacteria, *Xcc* has been used most commonly for pathology research in the experimental model plant *Arabidopsis thaliana* (Buell, 2002), whereas the interactions between *Xcr* and *A. thaliana* remain largely unexplored. While there is evidence that *Xcr* can infect *A. thaliana*, previous studies have been inconclusive with respect to phenotypic variation in responses of different accessions to specific strains. Early investigations used leaf spotting *X. campestris* strains assigned to the pathovars *raphani* and *armoraciae*. Most of these strains were non-pathogenic or only weakly pathogenic in the *A. thaliana* accessions tested (Davis *et al.*, 1991; Parker *et al.*, 1993); the pathotype strain of *Xcr* (NCPPB1946) was the only strain reported as pathogenic to several *A. thaliana* accessions, but differential responses to this strain were not identified (Parker *et al.*, 1993).

The starting point for the present study was a preliminary screen which identified two *A. thaliana* accessions (Col-0 and Nd-1) that exhibit distinct responses to a strain of *Xcr* race 2 (resistance and susceptibility, respectively) (E. Holub and J. Vicente, pers. comm.). A further survey of other accessions that show distinct phenotypic responses to *Xcr*, could provide useful sources for the identification of the genetic loci controlling resistance to *Xcr*. Once accessions showing different phenotypes are identified, then populations derived from these accessions can be used for genetic studies.

Thus, the preliminary observation that two *A. thaliana* accessions showed distinct responses to *Xcr* race 2 together with the availability of characterized strains of *Xcr* (Vicente *et al*., 2006), prompted the main aim of this Chapter: to characterize

the natural phenotypic variation of interactions in the largely unexplored *A. thaliana-Xcr* pathosystem, as a basis for investigating the molecular mechanisms of plant resistance to *Xcr*. The specific objectives were to:

a) Test different methods of inoculation of *A. thaliana* plants with *Xcr*.

b) Characterize the phenotypic variation in interactions between a standard set of *A. thaliana* accessions and representative strains of the three known *Xcr* races.

c) Generate comparative data for phenotypic variation of the same *A. thaliana* accessions in response to strains that represent three important races of *Xcc* (the two most common races in *Brassica oleracea*, races 1 and 4; and the broadly virulent race 6), and a *X. campestris* strain from Mauritius described to cause leaf spot and blight symptoms in cabbage (R. Lobin, pers. comm.).

## 2.2 MATERIALS AND METHODS

### 2.2.1 *Arabidopsis thaliana* accessions and growth conditions

A total of 100 *A. thaliana* accessions collected worldwide was chosen for this study (listed in Table 4) and comprised two distinct working collections, including:

a) Core collection: 22 accessions including the 19 parents of the Multi-parent Advanced Generation Inter-Cross ('MAGIC') population described by Kover *et al.*, 2009, two parents (Col-5 and Nd-1) from a recombinant inbred population developed by Holub *et al.* (1994) and the accession Ws-3 (Borhan *et al.*, 2008).

b) Weigel collection: 88 accessions representative of the natural variation in the species from regions in Europe, Asia and Africa assembled by D. Weigel (Max Planck Institute, Tuebingen, Germany) for whole genome sequencing as part of the 1001 Genomes Project (http://1001genomes.org/; Cao *et al.*, 2011).

Seeds were obtained from a collection assembled at the University of Warwick (UK) by E. Holub. Seed samples were originally obtained from several sources including: P. Kover (University of Bath, UK; founder parents of the MAGIC recombinant inbred lines), J. Dangl (University of North Carolina, USA; accession Nd-1), D. Weigel (Max Planck Institute, Tuebingen, Germany; Weigel collection) and Lehle Seeds (http://www.arabidopsis.com; accessions Ws-3 and Col-5).

**Table 4**. List of *Arabidopsis thaliana* accessions used in this study, geographical origin and source of acquisition

| ***A. thaliana* accession** | | **Geographical origin** | **Source (reference)** |
|---|---|---|---|
| **Name** | **Alternative name** | | |
| ***Core collection*** | | | |
| Bur-0 | Burren | Ireland | Kover, P. (Kover *et al.*, 2009) |
| Can-0 | Canary Islands | Canary Islands | Kover, P. (Kover *et al.*, 2009) |
| Col-0 | Columbia | USA | Kover, P. (Kover *et al.*, 2009) |
| Col-5 | Columbia *glabrous1* | na | Lehle Seeds (Holub *et al.*, 1994) |
| Ct-1 | Catania | Italy | Kover, P. (Kover *et al.*, 2009) |
| Edi-0 | Edinburgh | Scotland | Kover, P. (Kover *et al.*, 2009) |
| Hi-0 | Hilversum | Netherlands | Kover, P. (Kover *et al.*, 2009) |
| Kn-0 | Kaunas | Lithuania | Kover, P. (Kover *et al.*, 2009) |
| Ler-0 | Landsberg *erecta* | Germany | Kover, P. (Kover *et al.*, 2009) |
| Mt-0 | Martuba | Libya | Kover, P. (Kover *et al.*, 2009) |
| Nd-1 | Niederzenz | Germany | Dangl, J. (Holub *et al.*, 1994) |
| No-0 | Nossen | Germany | Kover, P. (Kover *et al.*, 2009) |
| Oy-0 | Oystese | Norway | Kover, P. (Kover *et al.*, 2009) |
| Po-0 | Poppelsdorf | Germany | Kover, P. (Kover *et al.*, 2009) |
| Rsch-4 | Rschew | Russia | Kover, P. (Kover *et al.*, 2009) |
| Sf-2 | San Feliu | Spain | Kover, P. (Kover *et al.*, 2009) |
| Tsu-0 | Tsushima | Japan | Kover, P. (Kover *et al.*, 2009) |
| Wil-2 | Wilna | Russia | Kover, P. (Kover *et al.*, 2009) |
| Ws-0 | Wassilewskija | Russia | Kover, P. (Kover *et al.*, 2009) |
| Ws-3 | Wassilewskija | Russia | Lehle Seeds (Borhan *et al.*, 2008) |
| Wu-0 | Wurzburg | Germany | Kover, P. (Kover *et al.*, 2009) |
| Zu-0 | Zurich | Switzerland | Kover, P. (Kover *et al.*, 2009) |
| ***Weigel collection*** | | | |
| Bak-2 | Bakuriani | Georgia | D. Weigel (Cao *et al.*, 2011) |
| Bak-7 | Bakuriani | Georgia | D. Weigel (Cao *et al.*, 2011) |
| Bay-0 | Bayreuth | Germany | D. Weigel |
| Bla-1 | Blanes | Spain | D. Weigel |
| Cdm-0 | Caldas de Miravete | Spain | D. Weigel (Cao *et al.*, 2011) |
| Cvi-0 | Cape Verde Islands | Cape Verde Islands | D. Weigel |
| C24 | Coimbra | Portugal | D. Weigel |
| Del-10 | Deliblato | Serbia | D. Weigel (Cao *et al.*, 2011) |
| Dog-4 | Dogruyol | Turkey | D. Weigel (Cao *et al.*, 2011) |
| Est-1 | Estland | Russia | D. Weigel |
| Ey-1.5.11 | na | Germany | D. Weigel (Bomblies *et al.*, 2010) |
| Ey-2 | na | Germany | D. Weigel (Bomblies *et al.*, 2010) |
| Ey-4 | na | Germany | D. Weigel (Bomblies *et al.*, 2010) |
| Ey15-2 | Eyach | Germany | D. Weigel (Cao *et al.*, 2011) |
| Ey-20 | na | Germany | D. Weigel (Bomblies *et al.*, 2010) |
| Fei-0 | Santa Maria da Feira | Portugal | D. Weigel (Cao *et al.*, 2011) |
| Haes-1 | na | na | D. Weigel |
| Haes-6 | na | Germany | D. Weigel and Sang-Tae Kim |
| Hh-0 | Hohenlieth | Germany | D. Weigel |

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**Table 4** (continued)

| *A. thaliana* accession | | Geographical origin | Source (reference) |
|---|---|---|---|
| **Name** | **Alternative name** | | |
| ***Weigel collection*** | | | |
| HKT2.4 | Heiligkreuztal 2 | Germany | D. Weigel (Cao *et al.*, 2011) |
| ICE1 | Bolin-1 | Romania | D. Weigel (Cao *et al.*, 2011) |
| ICE7 | Lecho-1 | Bulgaria | D. Weigel (Cao *et al.*, 2011) |
| ICE29 | Slavi-1 | Bulgaria | D. Weigel (Cao *et al.*, 2011) |
| ICE33 | Jablo-1 | Bulgaria | D. Weigel (Cao *et al.*, 2011) |
| ICE36 | Dobra-1 | Serbia | D. Weigel (Cao *et al.*, 2011) |
| ICE50 | Toufl-1 | Morocco | D. Weigel (Cao *et al.*, 2011) |
| ICE60 | Stepn-2 | Russia | D. Weigel (Cao *et al.*, 2011) |
| ICE61 | Stepn-1 | Russia | D. Weigel (Cao *et al.*, 2011) |
| ICE63 | Copac-1 | Russia | D. Weigel (Cao *et al.*, 2011) |
| ICE70 | Borsk-2 | Russia | D. Weigel (Cao *et al.*, 2011) |
| ICE71 | Shigu-1 | Russia | D. Weigel (Cao *et al.*, 2011) |
| ICE72 | Shigu-2 | Russia | D. Weigel (Cao *et al.*, 2011) |
| ICE73 | Kidr-1 | Russia | D. Weigel (Cao *et al.*, 2011) |
| ICE75 | Krazo-2 | Russia | D. Weigel (Cao *et al.*, 2011) |
| ICE79 | Voeran-1 | Italy | D. Weigel (Cao *et al.*, 2011) |
| ICE92 | Angit-1 | Italy | D. Weigel (Cao *et al.*, 2011) |
| ICE93 | Apost-1 | Italy | D. Weigel (Cao *et al.*, 2011) |
| ICE97 | Ciste-1 | Italy | D. Weigel (Cao *et al.*, 2011) |
| ICE98 | Ciste-2 | Italy | D. Weigel (Cao *et al.*, 2011) |
| ICE102 | Galdo-1 | Italy | D. Weigel (Cao *et al.*, 2011) |
| ICE104 | Lago-1 | Italy | D. Weigel (Cao *et al.*, 2011) |
| ICE106 | Mammo-1 | Italy | D. Weigel (Cao *et al.*, 2011) |
| ICE107 | Mammo-2 | Italy | D. Weigel (Cao *et al.*, 2011) |
| ICE112 | Moran-1 | Italy | D. Weigel (Cao *et al.*, 2011) |
| ICE119 | Timpo-1 | Italy | D. Weigel (Cao *et al.*, 2011) |
| ICE120 | Valsi-1 | Italy | D. Weigel (Cao *et al.*, 2011) |
| ICE127 | Kly1 | Russia | D. Weigel (Cao *et al.*, 2011) |
| ICE130 | Kly4 | Russia | D. Weigel (Cao *et al.*, 2011) |
| ICE134 | Koz2 | Russia | D. Weigel (Cao *et al.*, 2011) |
| ICE138 | Leb-3 | Russia | D. Weigel (Cao *et al.*, 2011) |
| ICE150 | Sij1 | Kazakhstan | D. Weigel (Cao *et al.*, 2011) |
| ICE152 | Sij2 | Kazakhstan | D. Weigel (Cao *et al.*, 2011) |
| ICE153 | Sij4 | Kazakhstan | D. Weigel (Cao *et al.*, 2011) |
| ICE163 | Altenb-2 | Italy | D. Weigel (Cao *et al.*, 2011) |
| ICE169 | Bozen-1-1 | Italy | D. Weigel (Cao *et al.*, 2011) |
| ICE173 | Bozen-1-2 | Italy | D. Weigel (Cao *et al.*, 2011) |
| ICE181 | Mitterberg-1 | Italy | D. Weigel (Cao *et al.*, 2011) |
| ICE212 | Castelfed-4-1 | Italy | D. Weigel (Cao *et al.*, 2011) |
| ICE213 | Castelfed-4-2 | Italy | D. Weigel (Cao *et al.*, 2011) |
| ICE216 | Rovero-1 | Italy | D. Weigel (Cao *et al.*, 2011) |
| ICE226 | Vezzano-2-1 | Italy | D. Weigel (Cao *et al.*, 2011) |
| ICE228 | Vezzano-2-2 | Italy | D. Weigel (Cao *et al.*, 2011) |

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**Table 4** (continued)

| *A. thaliana* accession | | Geographical origin | Source (reference) |
|---|---|---|---|
| **Name** | **Alternative name** | | |
| ***Weigel collection*** | | | |
| Kastel-1 | Kastel Mountain | East Europe | D. Weigel (Cao *et al.*, 2011) |
| Koch-1 | Kocherov | Ukraine | D. Weigel (Cao *et al.*, 2011) |
| Lerik1-3 | Lerik | Azerbaijan | D. Weigel (Cao *et al.*, 2011) |
| Mer-6 | Merida | Spain | D. Weigel (Cao *et al.*, 2011) |
| Nemrut-1 | Nemrut | Turkey | D. Weigel (Cao *et al.*, 2011) |
| Nie1-2 | Niederreutin | Germany | D. Weigel (Cao *et al.*, 2011) |
| Ped-0 | Pedriza | Spain | D. Weigel (Cao *et al.*, 2011) |
| Pra-6 | Pradena del Rincon | Spain | D. Weigel (Cao *et al.*, 2011) |
| Qui-0 | Quintela | Spain | D. Weigel (Cao *et al.*, 2011) |
| Rue3.1-31 | na | Germany | D. Weigel (Cao *et al.*, 2011) |
| Sha | Shahdara | Tajikistan | D. Weigel (Cao *et al.*, 2011) |
| Star-8 | Starzach | Germany | D. Weigel (Cao *et al.*, 2011) |
| Tsu-1 | Tsushima | Japan | D. Weigel (Ossowski *et al.*, 2008) |
| TueSB30-3 | na | Germany | D. Weigel (Cao *et al.*, 2011) |
| Tuescha-9 | Tuebingen - Schaal | Germany | D. Weigel (Cao *et al.*, 2011) |
| TueUM1 | na | Germany | D. Weigel and Sang-Tae Kim |
| TueV13 | na | Germany | D. Weigel (Cao *et al.*, 2011) |
| TueWa1-2 | Tuebingen - Wanne | Germany | D. Weigel (Cao *et al.*, 2011) |
| Uk-1 | Umkirch | Germany | D. Weigel |
| Uk-3 | Umkirch | Germany | D. Weigel |
| Vash-1 | Vashlovani | Georgia | D. Weigel (Cao *et al.*, 2011) |
| Vie-0 | Vielha | Spain | D. Weigel (Cao *et al.*, 2011) |
| WalhaesB4 | na | Germany | D. Weigel (Cao *et al.*, 2011) |
| Wen-2 | na | na | D. Weigel and Sang-Tae Kim |
| Xan-1 | Xanbulan | Azerbaijan | D. Weigel (Cao *et al.*, 2011) |
| Yeg-1 | Yeghegis | Armenia | D. Weigel (Cao *et al.*, 2011) |

Abbreviation: na, not available.

*A. thaliana* seeds were suspended in sterile 0.1% (w/v) agarose solution and stored for 3-5 days in the dark at −4 °C. This cold treatment promotes and synchronises seed germination (Yamauchi *et al.*, 2004). Trays fitted with P40 modules composed of 40×38×52 mm pots (Vacapot 40, BHGS) were filled with a 6:1:1 compost mixture of commercial peat-based compost Levington's F2S, fine sand and vermiculite (Holub *et al.*, 1994) treated with Intercept 5GR (Everris; 0.28 g/dm$^3$ of compost). Trays filled with compost mix were water-soaked, and cold treated seeds were sown on the compost surface by dispensing three to five seeds per pot using a disposable pipette. Trays were covered with lids and placed in a growth room at 20 °C under a photoperiod of 10 h at a light intensity of approximately 100 µmol/m$^2$s$^1$. Lids were removed 10 days after sowing and extra seedlings were removed to leave only one seedling per pot.

### 2.2.2 Bacterial strains

Twenty *X. campestris* strains were studied in this Chapter (Table 5) including:

a) Fifteen *Xcr* strains (5 strains representative of each race): HRI6490, 6489, 6491, 7979 and 8298 (race 1); HRI6497, 6518, 6520, 8305 and 8474 (race 2); and HRI6492, 6519, 8299, 8307 and 8503 (race 3).

b) Three race-type strains of important races of *Xcc*: HRI3811 (race 1), 1279A (race 4) and 6181 (race 6). *Xcc* races 1 and 4 were selected because they are the most common races in *B. oleracea* crops worldwide and *Xcc* race 6 shows broad virulence in the brassica differentials used to discriminate *Xcc* races (Vicente & Holub, 2013).

c) A *X. campestris* strain (HRI8506) from Mauritius reported to cause leaf spot and blight symptoms in cabbage (R. Lobin, pers. comm.).

**Table 5**. List of *Xanthomonas campestris* strains used for pathogenicity experiments in rosette plants of *Arabidopsis thaliana*

| HRI strain accession (other designation) | Isolation | | | Source or reference[a] |
|---|---|---|---|---|
| | Host | Geographical origin | Year | |
| ***Xanthomonas campestris* pv. *raphani*** | | | | |
| **Race 1** | | | | |
| 6489 (B-122) | na | Arizona, USA | 1984 | 3 |
| 6490$^{Rt}$ (P5034) | *Brassica oleracea* var. *botrytis* (cauliflower) | France | 1995 | 3 |
| 6491 (P5043) | *B. oleracea* var. *botrytis* (cauliflower) | France | 1995 | 3 |
| 7979 | *B. oleracea* var. *capitata* (cabbage) | Southern Hemisphere | 2000 | 3 |
| 8298 (0-71) | *Raphanus sativus* (radish) | Minnesota, USA | na | 3 |
| **Race 2** | | | | |
| 6497 (P5118) | na | na | 1997 | 3 |
| 6518 (TMR61) | *B. rapa* | Aichi, Japan | 1986 | 3 |
| 6520 (TMR137) | *B. rapa* var. *pekinensis* (Chinese cabbage) | Nagano, Japan | 1986 | 3 |
| 8305$^{Rt}$ (BR-25) | *B. rapa* var. *perviridis* (spinach mustard) | Oklahoma, USA | 1995 | 3 |
| 8474 (P764, NCPPB4451) | *Erysimum cheiri* (wallflower) | UK | na | NCPPB, D. Stead and J. Vicente |
| **Race 3** | | | | |
| 6492 (P5056) | *B. oleracea* var. *capitata* (cabbage) | France | 1995 | 3 |
| 6519$^{Rt}$ (TMR74) | *R. sativus* (radish) | Shizuoka, Japan | 1985 | 3 |
| 8299 (DC91-2) | *Solanum lycopersicum* (tomato) | Ontario, Canada | na | 3 |
| 8307 (XLS6) | *B. oleracea* var. *capitata* (cabbage) | North Carolina, USA | na | 3 |
| 8503 (756C) | *B. oleracea* var. *capitata* (cabbage) | East Asia | na | 1 |
| ***X. campestris* pv. *campestris*** | | | | |
| **Race 1** | | | | |
| 3811$^{Rt}$ (PHW1205) | *B. oleracea* | USA | na | 2 |
| **Race 4** | | | | |
| 1279A$^{Rt}$ | *B. oleracea* var. *capitata* (cabbage) | Cornwall, UK | 1984 | 2 |
| **Race 6** | | | | |
| 6181$^{Rt}$ (Xcc551) | *B. rapa* | Sardoal, Portugal | 1996 | 2 |
| ***X. campestris*** | | | | |
| 8506[b] | *B. oleracea* var. *capitata* (cabbage) | Mauritius | 2009 | R. Lobin |

Abbreviations: HRI, formerly Horticulture Research International, now University of Warwick Crop Centre, Wellesbourne, UK; na, not available; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, UK; Rt, race-type strain.

[a] References: 1, Bogdanove *et al.* (2011); 2, Vicente *et al.* (2001); 3, Vicente *et al.* (2006).

[b] *X. campestris* strain described to cause leaf spot and blight symptoms in cabbage (R. Lobin, pers. comm.).

### 2.2.3 Inoculum preparation

Bacterial strains were streaked from nutrient broth 15% (v/v) glycerol stocks stored at −76 °C onto King's B medium plates (King *et al*., 1954; Appendix 2) and grown for 48 h at 28 °C. Bacterial suspensions were prepared in sterile distilled water. All suspensions were filtered through cotton gauze to remove the excess of xanthan gum. The concentration of bacteria was adjusted to approximately $2\times10^8$ colony-forming units (cfu)/ml by measuring the absorbance (A) of the bacterial suspensions at 640 nm using a spectrophotometer (Spectronic 21, Milton Roy Company) and adjusting it to $A_{640nm}$ 0.2. The estimation of the $A_{640nm}$ 0.2 as a measure for the bacterial concentration of $2\times10^8$ cfu/ml is presented in Appendix 3.

### 2.2.4 Plant inoculations

All inoculations were carried out on four-week-old plants. Plant inoculations were carried out using two different methods, spraying of entire plants and wounding of individual leaves. In spray inoculations, the surfactant Silwet L-77 (Lehle Seeds) was added to bacterial suspensions just before inoculation at a concentration of 0.05% (v/v). Plants were sprayed using a DeVilbiss Atomizer 15 (DeVilbiss Health Care) connected to a pump (Huvema) at a pressure of 25 psi. The nozzle was kept at least 15 cm away from the plants to avoid mechanical damage of the plant tissues. This method was adapted from methods previously described by Katagiri *et al.* (2002) and Vicente *et al.* (2006). In wound inoculations, no surfactant agents were added to the bacterial suspensions. The four youngest adult leaves of each plant were selected for inoculation and the midvein of each leaf was pierced in three points with a sterile pin previously dipped in a bacterial suspension as described by Meyer *et al.* (2005).

After inoculation, plants were maintained in growth chambers at 22 °C with a photoperiod of 10 h and light intensity of 100 $\mu mol/m^2s^1$. Plants were covered with clear plastic bags during the first 24 h after inoculation to provide high humidity conditions.

### 2.2.5 Assessment of interaction phenotypes

The interactions between *A. thaliana* accessions and the bacterial strains under study were evaluated based on disease symptoms expression. Plants were examined regularly since the day of inoculation to follow the symptoms development. Scoring scales were then developed to rate the interaction phenotypes observed following each of the inoculation methods. The interactions phenotypes with *Xcr* strains were scored 10 days after inoculation (dai) and the interaction phenotypes with *Xcc* strains and the *X. campestris* strain HRI8506 were scored 14 dai.

### 2.2.6 Phenotyping experiments

Plant inoculations were carried out in three main experiments as follows:

a) Twenty-two *A. thaliana* accessions (core collection, Table 4) were inoculated with seven *X. campestris* strains that included the type strains of the three known races of *Xcr* (race 1, HRI6490; race 2, HRI8305; race 3, HRI6519), type strains of *Xcc* races 1, 4 and 6 (HRI3811, 1279A and 6181, respectively) and the *X. campestris* strain HRI8506 (Table 5). Plants were inoculated by spray and wound methods with all strains except for inoculations with the type strains of *Xcc* races 1 and 4 that were only performed with the wound method. For each inoculation method, four plants of each accession were tested with each strain in two experiment repeats.

b) A selection of ten *A. thaliana* accessions that showed distinct interaction phenotypes with the type strains of each *Xcr* race was further examined with four additional representative strains of each race. The *A. thaliana* accessions selected were: Col-5, Ct-1, Kn-0, Nd-1, Oy-0, Po-0, Rsch-4, Wil-2, Ws-0 and Zu-0 (Table 4). The *Xcr* strains tested were: HRI6489, 6491, 7979 and 8298 (race 1); HRI6497, 6518, 6520 and 8474 (race 2); and HRI6492, 8299, 8307 and 8503 (race 3) (Table 5). Two plants of each accession were spray-inoculated with each strain in one experiment repeat.

c) Eighty-eight *A. thaliana* accessions that comprised the Weigel collection (Table 4) were inoculated with the type strain of *Xcr* race 2 (HRI8305). Two plants per accession were spray-inoculated in one experiment along with the accessions Nd-1 and Col-0 that were used as controls.

In each experiment repeat, a set of *A. thaliana* accessions was inoculated by spray with a mock inoculum composed of sterile distilled water with 0.05% (v/v) Silwet L-77 or by wounding with sterile distilled water.

## 2.3 RESULTS

### 2.3.1 Symptom development of *Xanthomonas campestris* strains in *Arabidopsis thaliana*

Distinct disease symptoms were observed following spray and wound inoculations of several *A. thaliana* accessions among the 22 accessions tested (core collection, Table 4) with three *Xcr* strains (HRI6490, 8305 and 6519), three *Xcc* strains (HRI1279A, 3811 and 6181) and a *X. campestris* strain from Mauritius reported to cause leaf spot and blight symptoms in cabbage (HRI8506; pers. comm., R. Lobin). No disease symptoms were observed on mock-inoculated control plants.

The *Xcr* strains produced typical leaf spot symptoms in spray-inoculated plants, mainly characterized by circular leaf spots and dark elongated lesions on the leaf petioles and midveins (Figures 2a,b). Leaf spots appeared at first water-soaked 3-5 days after inoculation (dai); and gradually became necrotic, pale-beige approximately 7-10 dai (Figures 2a-e). Spots were not limited by leaf veins and in some cases appeared slightly sunken (Figure 2c), cracked and perforated (Figures 2d,e). Enlargement and coalescence of leaf spots was frequently observed forming irregular necrotic lesions (Figures 2f,g). In some cases, water-soaked tissue around leaf spots was observed followed by necrosis of the same tissue (Figure 2g). In wound-inoculated plants, *Xcr* strains caused pale-beige necrotic lesions surrounding the wound inoculation site visible 5-7 dai and, in some cases, lesions enlarged along the midvein (Figure 2h), but rarely led to leaf collapsing.

In contrast, the *Xcc* strains produced typical black rot symptoms. In spray-inoculated plants, lesions started at the leaf margin and gradually enlarged into chlorotic V-shaped lesions that frequently became necrotic, and darkened veins were observed in some accessions (*e.g.* Ler-0 or Mt-0) (Figures 3a,b). Chlorotic/necrotic V-shaped lesions were also observed in wound-inoculated plants, starting from the inoculation sites in the midvein of the leaf (Figures 3c,d). Symptoms were first observed around 7-10 dai with either inoculation method. The *X. campestris* strain HRI8506 from Mauritius produced symptoms that were identical to those incited by the *Xcc* strains tested.

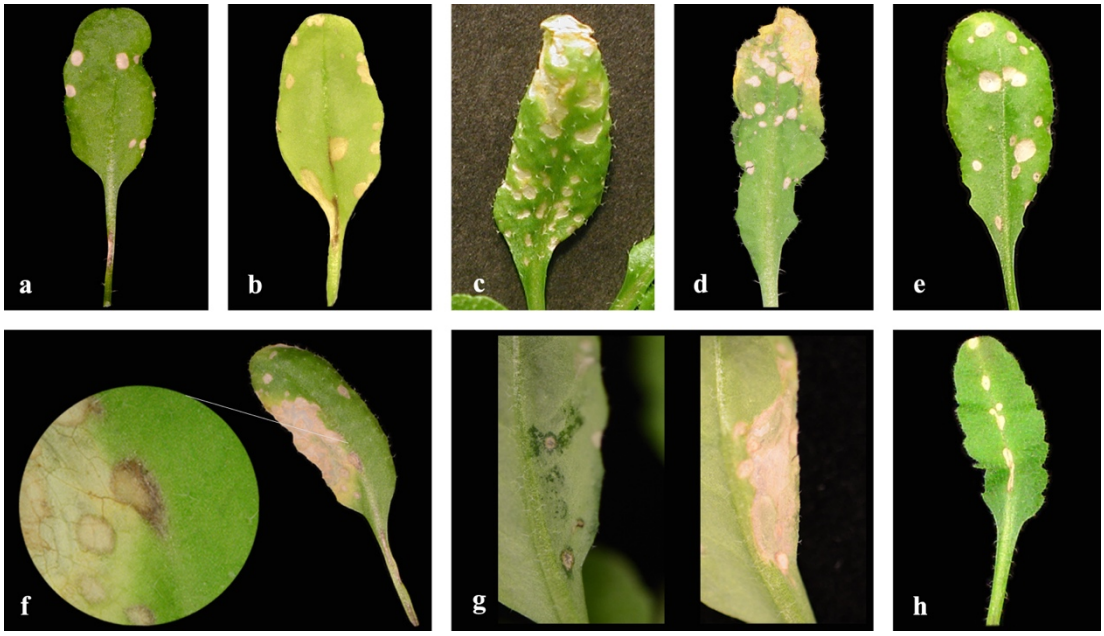



**Figure 2**. Symptoms induced by *Xanthomonas campestris* pv. *raphani* on spray-inoculated (a-g) and wound-inoculated (h) *Arabidopsis thaliana* leaves. **a**, circular spots and dark elongated lesion in the petiole 10 days after inoculation (dai); **b**, dark elongated lesion in the midvein 10 dai; **c**, sunken necrotic enlarged spots 7 dai; **d-e**, cracked and perforated spots 14 dai; **f**, close up of leaf spots and necrotic lesions not limited by leaf veins 15 dai; **g**, water-soaked tissue surrounding spots 9 dai (left) followed by necrosis of the tissue 7 days later (right); **h**, wound inoculated leaf with elongated necrotic lesions around the inoculation site and along the midvein 10 dai.


**Figure 3**. Symptoms induced by *Xanthomonas campestris* pv. *campestris* on spray-inoculated (a, b) and wound-inoculated (c, d) *Arabidopsis thaliana* leaves. **a**, chlorotic V-shaped lesion 14 days after inoculation (dai); **b**, close up of necrotic lesion and darkened veins 11 dai; **c**, chlorotic V-shaped lesion initiated from the inoculation site in the midvein 14 dai; **d**, V-shaped lesion showing necrosis in the leaf margin 14 dai.

### 2.3.2 Evaluation of the interaction phenotypes of *Arabidopsis thaliana* and *Xanthomonas campestris*

Six-point scales were defined to assess the disease symptom expression observed in the interactions between *A. thaliana* accessions and *X. campestris* strains with each of the inoculation methods used (spray and wound). The scales presented in Figure 4 and Figure 5 were defined for scoring the interaction phenotypes observed in plants inoculated with *Xcr* strains by spray and wound inoculation methods, respectively. The scales presented in Figure 6 and Figure 7 were defined for scoring the interaction phenotypes observed in plants inoculated with *Xcc* strains and the *X. campestris* strain HRI8506 by spray and wound inoculation methods, respectively.

The phenotypic scales were defined based on the leaf area expressing disease symptoms and the type of lesions observed. Overall, scores 0 or 1 describe absence of typical disease symptoms or only minor lesions, respectively; score 2 describes lesions restricted to a small area of the leaf; and scores 3 to 5 describe development of typical disease symptoms.

Each inoculated plant was scored with the maximum score observed among inoculated adult leaves. In spray inoculations, entire plants were sprayed and plants were scored with the maximum score observed among all adult leaves. In wound inoculations, all inoculated leaves per plant were individually scored and each plant replicate was given the maximum score observed among the four inoculated leaves.


**Figure 4**. Phenotype scale for scoring symptoms caused by *Xanthomonas campestris* pv. *raphani* in spray-inoculated *Arabidopsis thaliana* leaves, 10 days after inoculation. **0**, no symptoms; **1**, confined tiny lesions; **2**, few leaf spots affecting no more than 5% of the leaf area; **3**, typical leaf spots affecting 5-25% of the leaf area; **4**, leaf spots and chlorosis affecting 25-50% of the leaf area; **5**, leaf spots and chlorosis affecting more than 50% of the leaf area.


**Figure 5**. Phenotype scale for scoring symptoms caused by *Xanthomonas campestris* pv. *raphani* in wound-inoculated *Arabidopsis thaliana* leaves, 10 days after inoculation. **0**, no symptoms; **1**, minor scar around the wound site; **2**, small necrosis surrounding the wound site; **3**; expanded necrosis throughout the midvein; **4**, necrosis and chlorosis affecting less than 50% of the leaf area; **5**, necrosis and chlorosis affecting at least 50% of the leaf area.


**Figure 6**. Phenotype scale for scoring symptoms caused by *Xanthomonas campestris* pv. *campestris* in spray-inoculated *Arabidopsis thaliana* leaves, 14 days after inoculation. **0**, no symptoms; **1**, small weak chlorosis in the leaf margin; **2**, small V-shaped lesion affecting less than 10% of the leaf area; **3,** typical V-shaped lesion affecting 10-25% of the leaf area; **4**, lesion affecting 25-50% of the leaf area; **5**, expanded lesion affecting more than 50% of the leaf area.


**Figure 7**. Phenotype scale for scoring symptoms caused by *Xanthomonas campestris* pv. *campestris* in wound-inoculated *Arabidopsis thaliana* leaves, 14 days after inoculation. **0**, no symptoms; **1**, weak chlorosis confined to the wound site; **2**, small V-shaped lesion affecting less than 10% of the leaf area; **3**, typical V-shaped lesion affecting 10-25% of the leaf area; **4**, lesion affecting 25-50% of the leaf area; **5**, expanded lesion affecting more than 50% of the leaf area.

### 2.3.3 Interactions between twenty-two *Arabidopsis thaliana* accessions and *Xanthomonas campestris* strains

Phenotypic variation was observed in interactions between 22 *A. thaliana* accessions (core collection, Table 4) and each type strain of *Xcr* races 1, 2 and 3 (HRI6490, 8305, 6519), *Xcc* races 1, 4 and 6 (HRI3811, 1279A, and 6181) and the *X. campestris* strain HRI8506. The maximum and the minimum (or unique) phenotype scores observed among four plant replicates within each combination of *A. thaliana* accession, bacterial strain and inoculation method used, are presented in Table 6.

The interaction phenotypes (IPs) were characterized according to the symptoms observed using different inoculation methods for different pathogens: spraying for interactions with *Xcr* strains and wounding for interactions with *Xcc* strains and the *X. campestris* strain HRI8506 (which produced symptoms identical to those caused by the *Xcc* strains). Spray inoculations with *Xcr* strains allowed reproducing typical leaf spot symptoms. Moreover, the spray method allowed to obtain more reproducible results than the wound method as some accessions exhibited leaf spot symptoms in all plant replicates tested by spray inoculation, but did not show symptoms in all wound-inoculated plants. Wound-inoculations with *Xcc* strains and strain HRI8506, allowed to easily identify each inoculated leaf and detect the vascular symptoms starting from the wounding sites. In sprayed plants with the *Xcc* race 6 strain and strain HRI8506, it was difficult to distinguish between disease symptoms and natural senescence of older leaves, particularly in the accessions Bur-0, Can-0 and Ct-1.

**Table 6**. Interaction phenotypes of twenty-two *Arabidopsis thaliana* accessions and seven *Xanthomonas campestris* strains [a]

| | HRI strain accession | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Xcr* | | | | | | *Xcc* | | | | *Xc* | |
| | 6490 (r1) | | 8305 (r2) | | 6519 (r3) | | 3811 (r1) | 1279A (r4) | 6181 (r6) | | 8506 [b] | |
| *A. thaliana* | Inoculation method | | | | | | | | | | | |
| accession | s | w | s | w | s | w | w | w | s | w | s | w |
| Incompatible interaction phenotypes with all *Xcr* strains (resistance) | | | | | | | | | | | | |
| Col-0 | 0 | 0-1 | 0-1 | 0-1 | 0-1 | 0-1 | 0 | 0-1 | 1-5 | 3-4 | 0-1 | 0-1 |
| Col-5 | 0 | 0-1 | 0-1 | 0-1 | 0-1 | 0-1 | 0 | 0-1 | 0-3 | 3-4 | 0 | 0 |
| Kn-0 | 0 | 0-1 | 1 | 1 | 0-1 | 1 | 0 | 0-3 | 0-2 | 0-3 | 1-3 | 0-5 |
| Wil-2 | 0-1 | 0-1 | 0-1 | 0-1 | 0-1 | 1 | 0 | 0-1 | 0-1 | 0-1 | 0 | 0-1 |
| Compatible interaction phenotypes with all *Xcr* srains (susceptibility) | | | | | | | | | | | | |
| Nd-1 | 3-4 | 3 | 3-5 | 3-4 | 3-4 | 3-4 | 0-1 | 3-5 | 0-1 | 0-2 | 1-3 | 3-4 |
| Zu-0 | 3 | 1-3 | 3-4 | 4 | 3-4 | 3-4 | 0 | 1-4 | 0-2 | 2-5 | 2-4 | 0-2 |
| Differential interaction phenotypes with *Xcr* strains | | | | | | | | | | | | |
| Oy-0 | 0-1 | 0-1 | 0 | 0-1 | 3 | 3 | 3 | 0 | 0 | 0-1 | 0-1 | 0-1 |
| Ws-0 | 0-1 | 0-1 | 0-1 | 1 | 3 | 1-3 | 0-3 | 0-1 | 0-3 | 0-1 | 0-1 | 0-1 |
| Ct-1 | 0-1 | 0-1 | 3 | 1-3 | 3 | 1-3 | 0-2 | 0-2 | 2-3 | 0-2 | 2-4 | 2-3 |
| Po-0 | 0-1 | 0-1 | 3 | 1-4 | 3 | 3 | 0-3 | 3 | 3-4 | 3-4 | 3-4 | 3-4 |
| Rsch-4 | 0-1 | 0-1 | 3 | 1-4 | 3 | 2-3 | 0-2 | 0-2 | 0-2 | 0-3 | 0-2 | 0-2 |
| Inconclusive interaction phenotypes with at least one *Xcr* strain | | | | | | | | | | | | |
| Edi-0 | 0-1 | 0-1 | 0 | 0-1 | 2-4 | 1-3 | 0-1 | 3-5 | 0-2 | 0-5 | 0-2 | 0-1 |
| No-0 | 0-1 | 0-1 | 1 | 0-1 | 2 | 1-2 | 0 | 0-1 | 0 | 1-3 | 0-2 | 0-2 |
| Tsu-0 | 0-1 | 0-1 | 1 | 0-1 | 1-2 | 1 | 2 | 0-1 | 0-1 | 0-5 | 0 | 0-1 |
| Ler-0 | 0-1 | 0-1 | 1-5 | 1-5 | 0-1 | 1 | 3-5 | 3-5 | 2-5 | 5 | 5 | 4-5 |
| Bur-0 | 0-1 | 0-1 | 2 | 2-3 | 1-2 | 1-3 | 0-2 | 0-3 | 1-3 | 0-3 | 0-2 | 0-3 |
| Can-0 | 0 | 1 | 2 | 2-4 | 1-2 | 2-3 | 3-5 | 3-5 | 3-4 | 3-4 | 3-4 | 4-5 |
| Mt-0 | 0 | 0-1 | 1-2 | 0-2 | 1-2 | 0-1 | 0 | 0 | 0-5 | 3-5 | 0-1 | 0-1 |
| Ws-3 | 1 | 0-1 | 2 | 1-2 | 2 | 1-3 | 0 | 0-2 | 0-3 | 0-3 | 0-4 | 0-3 |
| Sf-2 | 1-2 | 0-1 | 3-5 | 3-4 | 3-4 | 3 | 0-5 | 0-4 | 0-2 | 0-2 | 2-5 | 0-2 |
| Hi-0 | 2-3 | 0-2 | 2-3 | 2-4 | 2 | 1-3 | 1-4 | 3-4 | 0-2 | 1-3 | 0-4 | 3 |
| Wu-0 | 1-2 | 0-1 | 2 | 1-2 | 2 | 1-2 | 0-1 | 1-3 | 0-2 | 0-3 | 0-3 | 0-2 |

Abbreviations: HRI, formerly Horticulture Research International, now University of Warwick Crop Centre, Wellesbourne, UK; na, not available; r, race; s, spray inoculation; w, wound inoculation; *Xc*, *Xanthomonas campestris*; *Xcc*, *X. campestris* pv. *campestris*; *Xcr, X. campestris* pv. *raphani*.

[a] The interaction phenotypes (IPs) are presented as follows: maximum and minimum scores of four plants examined in two experiment repeats, with each of the two inoculation methods, spray (s) and wound (w), 10 or 14 days after inoculation with *Xcr* or *Xcc* strains and *Xc* strain HRI8506, respectively; IPs were scored using a six-point scale (see Figures 4 to 7). IPs were characterized according to the results obtained by spray-inoculation for *Xcr* strains and wound-inoculation for *Xcc* strains and *Xc* strain HRI8506, as follows: incompatible (resistance; highlighted in green) when no symptoms were observed in any plant tested (IP 0 to 1); compatible (susceptibility; highlighted in yellow) when all plants tested showed typical disease symptoms (IP 3 to 5); inconclusive when, either at least one plant showed lesions restricted to a small area of the leaf (IP 2), or susceptible and resistant phenotypes were observed among individual plants (not highlighted).

[b] *X. campestris* strain from Mauritius reported to cause leaf spot and blight symptoms in cabbage (R. Lobin, pers. comm.).

The IPs were considered: incompatible (plant resistance) when no typical symptoms were observed among all plants tested (IP 0-1) and compatible (plant susceptibility) when typical disease symptoms affecting a substantial area of the leaf were observed in all plants tested (IP 3-5). The IPs were considered inconclusive when at least one plant showed lesions affecting a small area of the leaf (IP 2) or when compatible and incompatible phenotypes were observed among plant replicates. These restrictive criteria aimed to highlight the most clear compatible and incompatible interaction phenotypes that were reproducible in all plants tested.

In interactions with *Xcr* strains, a few accessions showed clearly distinct phenotypes as illustrated in Figure 8. Four accessions (Col-0, Col-5, Kn-0 and Wil-2) were resistant to all strains tested (incompatible interaction); two accessions (Nd-1 and Zu-0) were susceptible to all strains (compatible interaction); and five accessions (Oy-0, Ws-0, Ct-1, Po-0 and Rsch-4) showed differential responses to the three strains tested representative of each known *Xcr* race. The results suggested that the latter accessions might distinguish the three known races of *Xcr*. This hypothesis was investigated further in this Chapter by testing more strains of each race and the results are presented in Section 2.3.4. The remaining 11 accessions tested showed inconclusive results in interactions between at least one plant and at least one strain (Table 6).

In interactions with *Xcc* strains, Wil-2 was resistant to all strains tested and Can-0 and Ler-0 were susceptible to all strains tested (Table 6). Differential responses of individual accessions to different *Xcc* strains were also identified. Col-0, Col-5 and Mt-0 were resistant to all strains except to *Xcc* race 6 (strain HRI6181) that produced disease symptoms in these accessions. Nd-1, Oy-0 and Edi-0 showed differential responses to *Xcc* race 1 (strain HRI3811) and *Xcc* race 4 (strain HRI1279A). Nd-1 and Edi-0 were susceptible to HRI1279A, but resistant to HRI3811 while Oy-0 was resistant to all strains except to HRI3811. The *X. campestris* strain HRI8506 was similar to *Xcc* race 4 in the interactions with brassica differentials of *Xcc* races (according to the results presented in Chapter 4), but it differed from the type strain of *Xcc* race 4 (HRI1279A) in the interaction phenotype with the *A. thaliana* accession Edi-0. This accession was resistant to the *X. campestris* strain HRI8506 and susceptible to the type-strain of *Xcc* race 4 (Table 6).

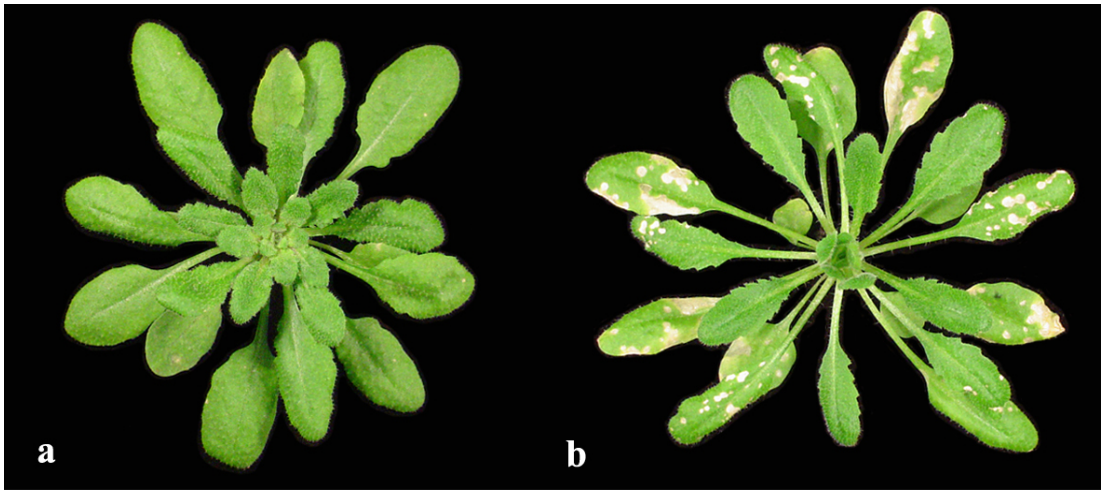



**Figure 8**. Differential interaction phenotypes of *Arabidopsis thaliana* accessions spray-inoculated with *Xanthomonas campestris* pv. *raphani* (strain HRI8305). Photographs were taken 10 days after inoculation: **a**, Col-0 incompatible phenotype (only minor lesions); **b**, Nd-1 compatible phenotype (typical leaf spot symptoms).

### 2.3.4 Differential responses of *Arabidopsis thaliana* accessions to races of *Xanthomonas campestris* pv. *raphani*

The results described above allowed the identification of ten *A. thaliana* accessions that showed either no typical symptoms (resistance) or development of typical symptoms (susceptibility) in interactions with all *Xcr* races, as well as accessions that showed clear differential responses to different *Xcr* races. In order to determine the usefulness of the genetic variation among *A. thaliana* accessions to identify sources of broad resistance and susceptibility to *Xcr*, and to distinguish among the three known *Xcr* races previously defined by differential reactions with brassica and radish lines (Vicente *et al.*, 2006), those accessions were tested for response to four additional strains of each *Xcr* race. All plants were spray-inoculated and the results obtained are presented in Table 7.

The *A. thaliana* accessions Col-5, Kn-0 and Wil-2, were resistant to all strains tested (IP 0-1) and the accessions Nd-1 and Zu-0 were susceptible (IP 3-5) (Table 7). Differential responses to *Xcr* races were observed with three accessions (Table 7). Oy-0 was resistant to all strains of *Xcr* races 1 and 2, but susceptible to all strains of *Xcr* race 3 whereas Po-0 and Rsch-4 were resistant to strains of *Xcr* race 1, but susceptible to strains of *Xcr* races 2 and 3. The remaining accessions (Ws-0 and Ct-1) showed an inconclusive phenotype with at least one strain (IP 2).

**Table 7**. Interaction phenotypes of twelve *Arabidopsis thaliana* accessions and five representative strains of the three known races of *Xanthomonas campestris* pv. *raphani* [a]

| | HRI strain no. | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *A. thaliana* | *Xcr* race 1 | | | | | *Xcr* race 2 | | | | | *Xcr* race 3 | | | | |
| accession | 6490[b] | 6489 | 6491 | 7979 | 8298 | 8305[b] | 6497 | 6518 | 6520 | 8474 | 6519[b] | 6492 | 8299 | 8307 | 8503 |
| Broad resistance | | | | | | | | | | | | | | | |
| Col-5 | 0 | 0 | 0 | 0 | 0 | 0-1 | 1 | 1 | 1 | 1 | 0-1 | 1 | 1 | 1 | 1 |
| Kn-0 | 0 | 0 | 0 | 0-1 | 0 | 1 | 1 | 0-1 | 1 | 1 | 0-1 | 0-1 | 1 | 1 | 1 |
| Wil-2 | 0-1 | 0 | 0 | 0 | 0 | 0-1 | 0 | 0-1 | 0 | 0 | 0-1 | 0 | 0 | 0 | 0 |
| Broad susceptibility | | | | | | | | | | | | | | | |
| Nd-1 | 3-4 | 5 | 3-4 | 4 | 4-5 | 3-5 | 5 | 4-5 | 4 | 3-4 | 3-4 | 3-4 | 4 | 4 | 4 |
| Zu-0 | 3 | 4 | 3 | 3-4 | 4 | 3-4 | 5 | 4 | 4 | 3 | 3 | 4 | 5 | 3 | 3 |
| Differential interactions between races | | | | | | | | | | | | | | | |
| Oy-0 | 0-1 | 0-1 | 0-1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 4 | 3 | 3 | 3 |
| Po-0 | 0-1 | 0-1 | 1 | 1 | 0 | 3 | 4 | 4-5 | 4 | 3-4 | 3 | 3 | 3 | 3 | 4 |
| Rsch-4 | 0-1 | 0 | 0 | 0 | 0-1 | 3 | 4-5 | 4 | 3 | 3 | 3 | 3-4 | 3 | 3 | 3 |
| Inconclusive interaction with at least one strain | | | | | | | | | | | | | | | |
| Ws-0 | 0-1 | 1 | 0 | 0 | 0 | 0-1 | 0 | 0 | 0 | 0 | 3 | 2 | 3 | 3 | 3 |
| Ct-1 | 0-1 | 0 | 1 | 0 | 0 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 4 |

Abbreviations: HRI, formerly Horticulture Research International, now University of Warwick Crop Centre, Wellesbourne, UK; no., number; *Xcr, Xanthomonas campestris* pv. *raphani*.

[a] The interaction phenotypes (IPs) presented were observed in two plants of each *A. thaliana* accession tested in one experiment repeat, 10 days after spray-inoculation; IPs were scored using a six-point scale presented in Figure 4. IPs were considered: incompatible (resistance; highlighted in green) when no symptoms were observed in any plant tested (IP 0 to 1); compatible (susceptibility; highlighted in yellow) when all plants tested showed typical disease symptoms (IP 3 to 5); inconclusive, when at plants showed limited development of leaf spots (IP 2; not highlighted).

[b] Interaction phenotypes of *A. thaliana* accessions with strains HRI6490, 8305 and 6519 are the same as those presented in Table 6.

### 2.3.5 Interactions between eighty-eight *Arabidopsis thaliana* accessions and the *Xanthomonas campestris* pv. *raphani* strain HRI8305

The *Xcr* strain HRI8305 (race 2) was chosen to explore the natural variation in defence response among an extensive collection of 88 *A. thaliana* accessions including several accessions with the genome sequenced or planned for sequencing in the 1001 Genomes Project (http://1001genomes.org/; Cao *et al.*, 2011) (Weigel collection listed in Table 4). This strain was selected because it was the strain used in the present study to investigate the genetic basis of resistance to *Xcr* in *A. thaliana* (Chapter 3).

A summary of the results obtained from spray inoculations with strain HRI8305 is given in Table 8. A total of 22 accessions showed no typical leaf spot symptoms (incompatible phenotype, IP 0-1) and 41 accessions showed typical leaf spot symptoms affecting a substantial area of the leaves (compatible phenotype,

IP 3-4). The remaining 25 accessions showed inconclusive phenotypes (IP 2 or IP 2-3).

**Table 8**. Interaction phenotypes of eighty-eight *Arabidopsis thaliana* accessions and the *Xanthomonas campestris* pv. *raphani* strain HRI8305 [a]

| IP | ***A. thaliana*** **accessions** (total number of accessions) |
|---|---|
| 0 | Hh-0, ICE130, ICE212, ICE63, Koch-1, Mer-6, Tsu-1, TueV13 (8) |
| 0-1 | C24, Haes-1, ICE120, ICE150, WalhaesB4, Yeg-1 (6) |
| 1 | Bak-2, ICE1, ICE127, ICE152, ICE213, ICE33, ICE72, ICE75 (8) |
| 3 | Bak-7, Ey-1.5.11, Ey-15.2, Ey-2, Ey-4, Ey-20, HKT2.4, ICE50, ICE61, ICE73, ICE104, ICE107, ICE119, ICE169, ICE173, ICE181, ICE226, Kastel-1, Lerik1-3, Qui-0, Tuescha-9, TueUM1, Vash-1, Wen-2 (24) |
| 3-4 | Del-10, ICE97, ICE98, Sha (4) |
| 4 | Cdm-0, Cvi-0, Dog-4, Haes-6, ICE7, ICE79, ICE92, ICE106, ICE112, ICE228, Rue3-1-31, Star-8, Tue-SB30-3 (13) |
| 2 | Bay-0, Bla-1, Est-1, Fei-0, ICE29, ICE36, ICE70, ICE71, ICE72, ICE102, ICE134, ICE138, ICE153, Nie-1.2, Pra-6, TueWa1-2, Uk-1, Uk-3, Vie-0, Xan-1 (19) |
| 2-3 | ICE60, ICE93, ICE163, ICE216, Nemrut-1, Ped-0 (6) |

Abbreviation: IP, interaction phenotype.

[a] The IPs presented were observed in two plants of each *A. thaliana* accession tested in one experiment repeat, 10 days after spray-inoculation; IPs were scored using a six-point scale presented in Figure 4. IPs were considered: incompatible (resistance; highlighted in green) when no symptoms were observed in any plant tested (IP 0 to 1); compatible (susceptibility; highlighted in yellow) when all plants tested showed typical disease symptoms (IP 3 to 5); inconclusive, when at least one plant showed limited development of leaf spots (IP 2; not highlighted).

## 2.4 DISCUSSION

### Inoculation methods

In this study, two different methods were tested for inoculation of *Xcr* in *A. thaliana*, including spraying of entire plants and wounding of leaves. *Xcr* was found to produce leaf spot symptoms in *A. thaliana* following spray inoculation, which are identical to those incited in natural plant hosts such as brassicas, radish and tomato as described by several authors (White, 1930; Tamura *et al.*, 1994; Vicente *et al.*, 2006). The abundance of leaf spots dispersed over the leaf area suggests that bacteria entered plants via stomata as in natural hosts. Therefore, the spray inoculation method seemed to allow for the natural stomata infecting behaviour of *Xcr*. As expected, the symptoms observed in plants that were spray-inoculated with *Xcr* were distinct from symptoms observed in plants inoculated with *Xcc* using the spray method. *Xcc* invades the plant host via hydathodes or wounds and colonizes primarily the xylem vessels causing typical black rot symptoms in *A. thaliana* and other *Brassicaceae* hosts (Simpson & Johnson, 1990; Lummerzheim *et al.*, 1993; Alvarez, 2000).

The wound method used in the present study was recommended by Meyer *et al.* (2005) to study the interactions between *Xcc* and *A. thaliana*. This method consists of piercing leaf veins with a bacterial suspension, and for *Xcc* it was shown to be a suitable method to reproduce its natural xylem invading behaviour and allow the development of V-shaped lesions as typically observed in natural field conditions (Meyer *et al.*, 2005). All *Xcc* strains tested in the present Chapter also produced typical black rot symptoms in some accessions as previously described by several authors (Simpson & Johnson, 1990; Buell, 2002; Meyer *et al.*, 2005). In inoculations with *Xcr*, disease symptoms were also observed using the wound method. Symptoms typically consisted of necrotic lesions along the leaf midvein and were similar to those described in brassicas and other plant host using a similar inoculation method (Vicente *et al.*, 2006). However, the wound method was laborious and time-consuming and, in some cases, the results were more variable than those obtained by spray inoculation for the same interactions. Furthermore, the wound method does not reflect the infection behaviour of *Xcr* through stomata and the spray method seems preferable to study the plant defence mechanisms associated to the

natural infecting behaviour of *Xcr*. The spray method is also suitable for large screening of plants because it allows rapid inoculation of several plants and scoring of interaction phenotypes.

Leaf infiltration of bacterial suspensions has been used as an inoculation method in previous studies for examining the interactions between *Xcr* and *A. thaliana* (Davis *et al.*, 1991; Parker *et al.*, 1993). However, these studies did not find differential responses to specific *Xcr* strains among the accessions tested and strains were reported either weakly/non-pathogenic or pathogenic to all accessions. It is possible that those results were influenced by the inoculation method used. The infiltration method places bacteria into the mesophyll tissue and thereby overcomes possible plant defence mechanisms that may prevent the natural entry of *Xcr* strains via stomata.

**Phenotypic variation in interactions between *Arabidopsis thaliana* and *Xanthomonas campestris* strains**

The results presented in this Chapter allowed identification of several *A. thaliana* accessions that showed distinct responses to the type strains of the three known races of *Xcr*. The results were then confirmed with additional strains of each race for selected accessions that showed reproducible phenotypes in all plants tested. Races of *Xcr* have been defined based on compatible and incompatible interactions with different brassica and radish genotypes (Vicente *et al.*, 2006) (Table 2, Chapter 1). The same races were discriminated based on interactions with specific *A. thaliana* accessions, but the interaction patterns differed for interactions with Oy-0. This accession was found resistant to *Xcr* races 1 and 2, but not race 3 and this pattern has not been observed with brassicas or radish genotypes. Most importantly, these results permitted to identify sources of resistance and susceptibility in *A. thaliana* to multiple *Xcr* strains as well as accessions that discriminate known races of *Xcr* (as summarized in Table 9), and provide a useful list of *A. thaliana* genotypes to further investigate the molecular basis of plant resistance to *Xcr*.

**Table 9**. Selected *Arabidopsis thaliana* accessions that showed contrasting interactions with races of *Xanthomonas campestris* pv. *raphani*

| | *Xanthomonas campestris* pv. *raphani* | | |
|---|---|---|---|
| *A. thaliana* accessions | race 1 | race 2 | race 3 |
| Col-0, Col-5, Kn-0, Wil-2 | − | − | − |
| Oy-0 | − | − | + |
| Po-0, Rsch-4 | − | + | + |
| Nd-1, Zu-0 | + | + | + |
| Number of strains tested | 5 | 5 | 5 |

+, compatible interaction (susceptibility); −, incompatible interaction (resistance).

In interactions between *A. thaliana* accessions and the type strains of the most common *Xcc* races (1 and 4) and the broadly virulent race 6, some accessions also showed clear differential responses to these strains in all plants tested (summarized in Table 10). Interestingly, Wil-2 was the only accession showing broad resistance to all *Xcr* and *Xcc* stains tested and Columbia (Col-0 and Col-5) was resistance to all *Xcr* and *Xcc* strains except to *Xcc* race 6. These results provide a useful list of accessions for future investigation of the molecular basis of plant resistance to *X. campestris*. The results obtained are in agreement with other studies that have described differential responses of *A. thaliana* accessions to *Xcc* strains (Simpson & Johnson, 1990; Daniels *et al.*, 1991; Lummerzheim *et al.*, 1993; Buell & Somerville, 1997; Meyer *et al.*, 2005; Guy *et al.*, 2013). In particular, the results agree with the results for interactions between the accession Columbia and the type strains of *Xcc* races 1, 4 and 6, recently presented by Guy *et al.* (2013). However, the results obtained in the present study provide a larger collection of accessions that show differential responses to these strains.

**Table 10**. Selected *Arabidopsis thaliana* accessions that showed contrasting interactions with races of *Xanthomonas campestris* pv. *campestris*

| | *Xanthomonas campestris* pv. *campestris* | | |
|---|---|---|---|
| *A. thaliana* accessions | race 1 | race 4 | race 6 |
| Wil-2 | − | − | − |
| Col-0, Col-5, Mt-0 | − | − | + |
| Oy-0 | + | − | − |
| Can-0, Ler-0 | + | + | + |
| Nd-1 | − | + | ? |
| Number of strains tested | 1 | 1 | 1 |

+, compatible interaction (susceptibility); −, incompatible interaction (resistance); ?, inconclusive.

The *X. campestris* strain HRI8506 was reported to cause leaf spot and blight symptoms in cabbage following an outbreak occurring in Mauritius (R. Lobin, pers. comm.). As shown in Chapter 4, this strain was similar to *Xcc* race 4 in the interactions with the standard brassica differentials used to discriminate *Xcc* races (Vicente & Holub, 2013) and produced symptoms more similar to those caused by *Xcc* than *Xcr* strains. In *A. thaliana,* it also produced similar symptoms to those produced by the *Xcc* strains and the interaction patterns with different accessions tested were similar to those observed with the type strain of *Xcc* race 4 except with the accession Edi-0. This accession was resistant to the strain from Mauritius, but susceptible to the type strain of *Xcc* race 4, and it provides an additional source of resistance to that strain.

# CHAPTER 3. Genetic characterization of resistance in *Arabidopsis thaliana* to *Xanthomonas campestris* pv. *raphani*

## 3.1 INTRODUCTION

In the previous Chapter, extensive phenotypic variation was described following inoculations of 22 accessions from a global collection of *Arabidopsis thaliana* with strains representing three known races of *Xanthomonas campestris* pv. *raphani* (*Xcr*). For example, two contrasting examples included the broadly susceptible accession Niederzenz (Nd-1) that exhibited full leaf spot symptoms in interactions with all three *Xcr* races, and the broadly resistant accessions Columbia (wild type Col-0 and the *glabrous-1* mutant Col-5) which exhibited no leaf spot symptoms in interactions with all three *Xcr* races. Other accessions showed differential responses to different *Xcr* races. The aim of the current Chapter was to investigate the genetic basis of this phenotypic variation.

Recombinant inbred lines (RILs) have been a powerful research tool for genetic mapping of loci that control natural phenotypic variation in *A. thaliana* (Koornneef *et al.*, 2004; Koornneef *et al.*, 2011). RILs have typically been generated from hand-pollinated outcrossing of two accessions. The resulting hybrid plants are then self-fertilized to produce a segregating $F_2$ population, and a sample of single seed descent lines are grown over six or more consecutive generations of self-fertilization to generate a population of plant lines that are nearly homozygous throughout the entire inbred genome. The homozygosity within each inbred line and recombinant variation among lines has practical advantages for genetic mapping. RILs can be propagated indefinitely and phenotyped in replicates for accurate prediction of causal parental allele(s) (Koornneef *et al.*, 2011). The multiple recombination events that occur in meiosis during the development of RILs promote the genetic variability among these lines, and the genome of each line will be unique for different combinations of chromosomal segments from the original parental lines (Jones *et al.*, 2009). Molecular markers that detect DNA sequence variation (*e.g.* point mutations and deletions or insertions) inherited from the parents can be used to genotype the inbreds and generate a detailed genetic linkage map of the five *A. thaliana* chromosomes for the population under study. The assessment of the association between allelic variation of molecular markers and phenotypic variation of a specific trait in an experimental population, provides a means to predict the genome position of the genetic loci that control a phenotypic trait (Koornneef *et al.*,

2004). This experimental procedure is referred to as interval mapping, and is the first key step towards molecular identification of genes that control the trait (*i.e.* map-based or positional cloning).

The first *A. thaliana* RIL populations were produced in the early 1990's (Reiter *et al.*, 1992; Lister & Dean, 1993). Since then, other *A. thaliana* RIL populations have been developed from two parent crosses (Koornneef *et al.*, 2011) and made available to the research community from Arabidopsis stock centres (The European Arabidopsis Stock Centre, http://arabidopsis.info/BrowsePage; Versailles Arabidopsis Stock Centre, http://dbsgap.versailles.inra.fr/vnat; and Arabidopsis Biological Resource Centre, https://abrc.osu.edu/). In addition, 19 *A. thaliana* accessions have been used to develop a Multi-parent Advanced Generation Inter-Cross (MAGIC) inbred population (Kover *et al.*, 2009). The MAGIC inbred population was produced by intermating the 19 parent accessions for four generations, before producing inbred lines from six generations of single seed descent. These lines therefore capture more genetic variation than RILs generated from two parents, as their genotypes are composed of allelic variation and distinct mosaics of genomic regions of the multiple founder parents. These lines and their founder parents have been genotyped for 1260 single nucleotide polymorphisms and a statistical methodology has been developed for mapping the genetic loci associated with phenotypic traits of interest (Kover *et al.*, 2009; http://mus.well.ox.ac.uk/magic/).

The objective of this Chapter was to determine the molecular basis of resistance in *A. thaliana* to at least one race of *Xcr*. A strain of *Xcr* race 2 (HRI8305) was chosen for mapping of major genetic loci controlling resistance in the MAGIC population. Resistance in the accession Columbia was targeted as an example of broad-spectrum resistance to all three *Xcr* races. Preliminary mapping had already identified a single locus on the bottom arm of chromosome 3 in Columbia for resistance to *Xcr* race 2 strain HRI8305 (E. Holub and J. Vicente, pers. comm.). In the current study, RILs generated from outcrossing of Columbia with the susceptible accession Niederzenz were used for fine-scale mapping, and a gene designated *RXCR1* was identified by loss-of-function and gain-of-function experiments that verified its role as the determinant of *Xcr* race 2 resistance.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Bacterial strains

The bacterial strains used in this Chapter included: one representative strain of each of the three known *Xcr* races (HRI6490, race 1; HRI8305, race 2 and HRI6519, race 3; details provided in Table 5, Chapter 2) that were used for phenotyping assays; and the *Agrobacterium tumefaciens* strain GV3101 (pMP90) (Koncz & Schell, 1986) that was used for genetic transformation of *A. thaliana*. The *Xcr* strains were maintained in Nutrient Broth (Difco) with 15% (w/v) glycerol at −76 °C. The *A. tumefaciens* strain GV3101 and transformed GV3101 strains, were maintained at the same temperature, in LB broth (Bent, 2006; Appendix 2) with 25% (w/v) glycerol.

### 3.2.2 Plant materials

The *A. thaliana* accessions used in this Chapter were as follows:

a) The standard laboratory accessions Columbia (Col-0), Columbia *glabrous-1* mutant (Col-5), Niederzenz (Nd-1) and Oystese (Oy-0). Seeds were obtained from E. Holub (University of Warwick, UK). The original source of each accession is given in Chapter 2 (Table 4).

b) A set of 353 Multi-parent Advanced Generation Inter-Cross (MAGIC) inbred lines derived from an intercrossing of 19 parental accessions (Kover *et al.*, 2009) (listed in Appendix 4). Seeds were purchased from NASC (Nottingham Arabidopsis Stock Centre, http://arabidopsis.info).

c) A set of 87 $F_9$ RILS selected from a mapping population derived from two parent crosses of accessions Columbia (Col-5 or Col-0) and Niederzenz (Nd-1) developed by Holub & Beynon (1997) (listed in Appendix 5). This set included: eight key recombinants within a map interval defined in this study for resistance to *Xcr* (3790, 3800, 3819, 3858, 3860, 3887, 3892 and 3893) selected from a publically available set of 98 lines that have been genotyped with molecular markers distributed across the genome (Deslandes *et al.*, 1998; Werner *et al.*, 2005; E. Holub, pers. comm.), and 79 additional lines which had not been

released publically (E. Holub, pers. comm.). Seeds were obtained from E. Holub (University of Warwick, UK).

d) An unpublished $F_9$ recombinant inbred mapping population of 98 lines derived from a two-parent cross of accessions Niederzenz (Nd-1) and Oystese (Oy-0), which was provided by E. Holub (University of Warwick, UK).

e) Six $F_8$ recombinant inbred lines (line number 7, 78, 107, 127, 219 and 167) selected from a mapping population derived from a cross Oy-0×Col-0 (Simon *et al.*, 2008). These were chosen as representative of different genotype combinations at the two loci mapped in this study associated to resistance to *Xcr* race 2 strain HRI8305. Seeds were purchased from Versailles Arabidopsis Stock Centre (http://publiclines.versailles.inra.fr; mapping population accession RV27).

f) 19 T-DNA insertion mutant lines of *A. thaliana* Col-0 (Alonso *et al.*, 2003) (listed in Appendix 6) with T-DNA insertions located in genes spanning a 62.31 kb interval mapped in the bottom arm of chromosome 3 of Columbia for resistance to *Xcr* race 2. They were selected using the T-DNA Express Arabidopsis Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress). Seeds were purchased from NASC (Nottingham Arabidopsis Stock Centre, UK; http://arabidopsis.info).

Plants were grown as described in Chapter 2 (Section 2.2.1) except for plants used for Agrobacterium-mediated transformation. For plant transformation, seeds were sown in 11 cm (diameter) × 9.5 cm (high) pots filled with compost into a dome shape. Nine plants were grown per pot and they were maintained in a glasshouse at 22 to 24 °C under a photoperiod of 16 h.

### 3.2.3 Phenotyping

Four-week-old *A. thaliana* rosettes were spray-inoculated with bacterial suspensions following the method described in Chapter 2, Section 2.2.5. The interaction phenotypes were scored 10 days after inoculation using the six-point scale developed in this study to assess symptoms caused by *Xcr* on spray-inoculated *A. thaliana* plants (Figure 4, Chapter 2).

The following plant lines were phenotyped for interaction with strain HRI8305 (*Xcr* race 2): 353 MAGIC lines (two plants tested per line in two experiment repeats); 12 $F_9$ Col×Nd-1 inbred lines that were identified as key recombinants for fine-mapping of a locus controlling resistance to *Xcr* race 2 on chromosome 3 of accession Columbia (lines 3790, 3800, 3819, 3858, 3860, 3887, 3892, 3893, 8011a, 8011b, 8044 and 8048); four plants per line tested in two experiment repeats); ten $F_1$ plants derived from each reciprocal cross of Col-5 and Nd-1; 1020 $F_2$ plants and 29 to 72 plants of each of 13 selected $F_3$ families, derived from a Col-5×Nd-1 cross; ten $F_1$ plants derived from each reciprocal cross of Nd-1 and Oy-0; 158 $F_2$ plants derived from a cross Nd-1×Oy-0; 98 $F_9$ Nd-1×Oy-0 recombinant inbred lines (three plants per line tested in one experiment); six $F_8$ lines derived from a cross Oy-0×Col-0 (four plants per line tested in one experiment); 19 Col-0 T-DNA insertion mutant lines (8 or 16 plants from homozygous or heterozygous lines respectively, tested in one experiment); and four to 58 plants of each of the 12 transgenic lines obtained in this study.

Two plant lines were also phenotyped for interaction with the type strain of each of the three *Xcr* races (HRI6490, race 1; HRI8305, race 2; HRI6519, race 3) and included: the $F_9$ Col-0×Nd-1 recombinant inbred 3790 and Col-0 T-DNA mutant SALK204228C (four plants per line tested in two experiment repeats).

At least two plants of resistant (Col-5 and/or Oy-0) and susceptible (Nd-1) accessions to *Xcr* race 2 were included as controls in every experiment.

### 3.2.4 Cross-pollination and self-pollination of *Arabidopsis thaliana* plants

Plants were grown until flowering. For cross-pollination, closed flower buds of a selected female parent were chosen. All the flower organs were removed except the carpel using fine-tip forceps. Anthers were removed from fully opened flowers of a selected male parent and the pollen was placed on the tip of the carpels of the female parent. The pollinated carpels were wrapped in cling film until siliques started to develop. Seeds were harvested from dried siliques.

For self-pollination, plants were covered with microperforated plastic bags before flowers opened to avoid cross-pollination. Seeds were collected from dried siliques.

### 3.2.5 DNA extraction

Genomic DNA was extracted from plant tissue using two methods, depending upon the required duration of storage for each sample.

For DNA samples required for long-term storage, extractions were performed using the DNeasy Plant kit (Qiagen) and following the manufacturer's instructions. The concentration and purity of the DNA was assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). DNA samples were stored at −20 °C.

The DNA samples required for only short-term storage were extracted using the REDExtract-N-Amp Plant PCR kit (Sigma-Aldrich) following the manufacturer's instructions. Leaf extracts were kept at 4 °C.

### 3.2.6 DNA amplification

Polymerase Chain Reaction (PCR) amplifications of genomic DNA were carried out using different reaction mixtures. For amplification of DNA samples extracted using the DNeasy Plant kit, the reaction mixtures contained 1× Biomix (Bioline), 0.5 µM of each primer and 2 ng/µl of DNA template. For amplification of DNA samples extracted with the REDExtract-N-Amp Plant PCR kit (Sigma-Aldrich), the reactions mixtures were performed in a total volume of 10 µl and contained 5 µl of REDExtract-N-Amp PCR ReadyMix, 0.5 µM of each primer and 2.5 µl of leaf disk extract.

PCR reactions were carried out using a touchdown PCR program (Korbie & Mattick, 2008) optimized in this study as follows: initial denaturation at 94 °C for 3 min; 10 cycles of 94 °C for 30 s, 65 to 56 °C (−1 °C per cycle) for 30 s, 72 °C for 1 min; 25 cycles of 94 °C for 30 s, 65 to 56 °C for 30 s, 72 °C for 1 min; and a final extension at 72 °C for 7 min. The PCR products were separated by electrophoresis on a 1 or 2% agarose gel (depending on the expected size of the DNA amplified

fragments) with 0.006% (v/v) GelRed nucleic acid gel stain (Biotium), at 100 to 80 V for 1 to 3 h. A volume of 6.25 µl of 1 kb Plus DNA ladder (50 ng/µl; Invitrogen) was loaded in at least one well of each gel to provide a reference for the DNA fragment size and concentration (25 ng/µl at 1650 bp band). Gels were visualized under UV light.

### 3.2.7 DNA sequencing and sequence analysis

PCR products were purified using the Qiaquick PCR Purification kit (Qiagen) according to the manufacturer's instructions. Purified PCR products were sequenced by a commercial service (GATC Biotech), in both directions with the forward and reverse primers used in PCR amplifications.

DNA sequences obtained for each sample were manually trimmed for quality by examination of the sequencing chromatogram peaks using the SeqMan program (Lasergene 9 software package, DNAstar). Sequences were aligned and compared using the same program.

### 3.2.8 Molecular markers and genotyping of *Arabidopsis thaliana* plants derived from crosses between Columbia and Niederzenz accessions

A search for DNA polymorphisms (point mutations or indels) between *A. thaliana* accessions Columbia and Nd-1 was performed within a 1.87 Mb interval (21.31 to 23.18 Mb) on chromosome 3, which was predicted to contain a source for resistance to *Xcr* race 2 strain HRI8305 in Columbia (as presented in Section 3.3.2). A total of 12 molecular markers were selected for genotyping of plants derived from crosses between Columbia (Col-5 or Col-0) and Nd-1 accessions, and used to resolve a narrower map interval. These markers are described in Table 11 and were selected as described below.

The genome sequence of the accession Columbia was scanned using the GBrowse tool (TAIR website; https://www.arabidopsis.org) for selection of genes spanning the target interval. Sequence similarity searches of gene sequences were performed in the TAIR10 database using the WU-BLAST tool (TAIR website;

https://www.arabidopsis.org/wublast/index2.jsp) to avoid selection of genes that occur in multiple copies throughout the genome. The Primer-BLAST online tool (Ye *et al.*, 2012; http://www.ncbi.nlm.nih.gov/tools/primer-blast) was used to design specific primers to the selected genes. Primers were then tested for amplification of gene fragments from Col-5 and Nd-1 as described in Section 3.2.6. Sequencing of PCR products and sequence analyses were performed as described in Section 3.2.7. Polymorphisms between Col-5 and Nd-1 were identified in the DNA sequences amplified with 12 primer pairs and were selected as molecular markers (listed in Table 11).

**Table 11**. Molecular markers, primers and restriction enzymes, used for genotyping *Arabidopsis thaliana* plants derived from crosses between accessions Columbia and Niederzenz

| Marker (Gene[a]) | Physical position in chromosome 3[b] (bp) | Primer | Sequence (5' to 3') | PCR product (bp) | Enzyme | *A. thaliana* accession: restriction fragments (bp) |
|---|---|---|---|---|---|---|
| m57530 | 21,298,021 - 21,298,772 | VP20-F | TGCAGAATTGCAATGGCCTCGT | 752/743[c] | AflIII | Col-5: 598; 154 |
| (At3g57530) | | VP20-R | ACCTTGGGCCATGGGTCCCT | | | Nd-1: 384; 214; 145 |
| m57660 | 21,358,413 - 21,359,880 | VP28-F | GGTCACCCGCCTTTCACCGT | 1468 | na | na |
| (At3g57660) | | VP28-R | CCCCCAGCTCTAGGGGACCA | | | |
| m57830 | 21,420,726 - 21,422,002 | VP26-F | GCGCCATTGGATGATGCGGC | 1277 | na | na |
| (At3g57830) | | VP26-R | AGCCCGAGCCTCAGGTGCAA | | | |
| m58090 | 21,512,640 - 21,513,847 | VP24-F | AGATACCGGGGGAGCCTGCG | 1208 | Fnu4HI | Col-5: 1193; 15 |
| (At3g58090) | | VP24-R | ACATGGACACGGTGCTGCCG | | | Nd-1: 780; 413; 15 |
| m58230 | 21,566,988 - 21,567,378 | VP22-F | ACCGTGGAGGTCAATGGCTTTCG | 391/390[c] | TaqI | Col-5: 194; 174; 23 |
| (At3g58230) | | VP22-R | TCCTAGCCCCACGTGCTTCCA | | | Nd-1: 193; 87; 87; 23 |
| m58310 | 21,584,271 - 21,584,761 | VP21-F | CTTGCTCCAGTGCCGGGGTG | 491 | BsmAI | Col-5: 357; 134 |
| (At3g58310) | | VP21-R | TGCGAGTAGCCTTTGCGGTCA | | | Nd-1: 491 |
| m58350 | 21,591,745 - 21,592,748 | VP17-F | TCGTTGGTGGCTGCAAATGGTAA | 1004/1003[c] | PleI | Col-5: 747; 169; 78; 10 |
| (At3g58350) | | VP17-R | AGCCCAGTCTCACTAGCCTCCT | | | Nd-1: 824; 169; 10 |
| m58470 | 21,626,858 - 21,628,060 | VP16-F | TGTGCGACGGGTTTTGATTTGTGC | 1203/1241[c] | BstUI | Col-5: 1107; 96 |
| (At3g58470) | | VP16-R | GCCGATGAGTGTTCCCTGCCA | | | Nd-1: 1203 |
| m59070 | 21,832,566 - 21,833,328 | VP14-F | CCACTGTTTCCGTTGATGTGGTCT | 763 | PacI | Col-5: 374; 200; 189 |
| (At3g59070) | | VP14-R | CGCCGCCGACCTTGTGATGA | | | Nd-1: 563; 200 |
| m59890 | 22,125,276 - 22,126,507 | VP11-F | CAAGTGGAAACCGAAAATGGCGG | 1232/1156[c] | RsaI | Col-5: 954; 141; 105; 32 |
| (At3g59890) | | VP11-R | GCTGCAAGAAACGCAACAACCTAGT | | | Nd-1: 566; 312; 141; 105; 32 |
| m60940 | 22,522,048 - 22,523,383 | VP7-F | TTCTGTGAATCGGAAACTGCTTCGT | 1336 | AflIII | Col-5: 541; 407; 388 |
| (At3g60940) | | VP7-R | GGGCCAGCTGGATCGGGGTA | | | Nd-1: 795; 541 |
| m62080 | 22,986,566 - 22,987,811 | VP3-F | CCTGTCCAGGTCTGGTCAATTGCT | 1246 | RsaI | Col-5: 1121; 125 |
| (At3g62080) | | VP3-R | TGCCAGCGCTGATTTCTTTGACC | | | Nd-1: 740; 381; 125 |

Abbreviations: bp, base pair; na, not available (for these markers, no enzymes were selected and plants were genotyped by sequencing).

[a] Gene identifier according to the genome sequence annotation in the TAIR website (The Arabidopsis Information Resource; https://www.arabidopsis.org).

[b] Physical positions in the AGI map of chromosome 3 of *A. thaliana* Columbia (AGI, 2000; TAIR website, https://www.arabidopsis.org; data source TAIR10).

[c] Expected size of PCR products of *A. thaliana* accessions Columbia and Niederzenz, respectively.

The software SeqBuilder (Lasergene 9 software package, DNAstar) was then used to select restriction enzymes that cleave the polymorphic DNA fragments from accessions Col-5 and Nd-1 into accession specific fragments. These enzymes were tested with the PCR products obtained from amplification of gene fragments from Col-5 and Nd-1. PCR products were digested with enzymes obtained from New England Biolabs or Promega, following the manufacturer's instructions. The enzymatic digestion products were separated by electrophoresis in 1 to 3% agarose gel for 1 to 3 hours depending on the expected size of the fragments.

The restriction enzymes that were confirmed to cleave the PCR products from Col-5 and Nd-1 into accession specific fragments (listed in Table 11) were then used for genotyping of plants derived from crosses between Columbia (Col-0 or Col-5) and Nd-1 accessions. This methodology was described by Konieczny & Ausubel (1993). For two markers (m57660 and m57830), no enzymes were selected and plants were genotyped by sequencing. In all genotyping procedures, DNA samples from Col-5 and Nd-1 were used as positive controls, and water was used as negative control.

### 3.2.9 Genotyping of *Arabidopsis thaliana* T-DNA insertion mutants

Eight plants of the *A. thaliana* Col-0 T-DNA mutant line SALK204228C (which showed loss-of-function for resistance to *Xcr* race 2 HRI8305) were genotyped by PCR to confirm the presence of the T-DNA insert as well as the homozygosity of the insertion. The T-DNA Express tool (http://signal.salk.edu/cgi-bin/tdnaexpress) was used to define the genotyping strategy and obtain the specific primers for the insert and flanking genomic regions (Table 12).

**Table 12**. Primers used for genotyping plants of the *Arabidopsis thaliana* Col-0 T-DNA mutant line SALK204228C

| PCR | Primer name | Sequence (5' - 3') | Expected fragments (bp) | | |
|---|---|---|---|---|---|
| | | | WT | HM | HT |
| 1 | LBb1.3 | ATTTTGCCGATTTCGGAAC | 0 | 458-758 | 458-758 |
| | RP | CTATGAATCAGCGAGGAGGTG | | | |
| 2 | LP | ACGAGTGATGACATTCCCAAG | 1069 | 0 | 1069 |
| | RP | Same as above | | | |

Abbreviations: WT, wild type (Col-0); HM, homozygous; HT, heterozygous.

Genomic DNA was extracted from each mutant plant and a Col-0 wild type plant, as described in Section 3.2.5. Two PCR amplifications were carried out for each DNA sample with each of the primer pairs: LP and RP which are specific for the genomic regions of Col-0 flanking the T-DNA insertion; and RP and LBb1.3 which is specific for the T-DNA insertion. The PCR amplifications were performed following the methods described in Section 3.2.6, except for the PCR program which was as follows: initial denaturation at 94 °C for 3 min, 25 cycles of 94 °C for 30 s, 55 for 30 s, 72 °C for 1 min; and a final extension at 72 °C for 7 min.

### 3.2.10 Inheritance and segregation analysis

In order to assess the inheritance of resistance of the accessions Col-5 and Oy-0 to the *Xcr* strain HRI8305, ten $F_1$ plants derived from reciprocal crosses of these resistant accessions and the susceptible accession Nd-1 were inoculated with this strain and the interaction phenotypes were scored as described in Section 3.2.3. All $F_1$ plants derived from crosses between Col-5 and Nd-1 were confirmed to be true hybrids by genotyping with the molecular marker m57350 (Table 11, Section 3.2.8) and observation on agarose gel of different size DNA fragments specific to each parent. The genotyping of $F_1$ plants derived from crosses between Oy-0 and Nd-1 was performed by U. Sonawala as part of her MSc research project (Sonawala, 2013).

The segregation of resistance to the *Xcr* strain HRI8305 was assessed in $F_2$ and $F_9$ plants derived from two parental crosses of Columbia (Col-0 or Col-5) or Oy-0 and Nd-1. Plants were inoculated with that strain and the interaction phenotypes were scored as described in Section 3.2.3. Phenotype data for 84 $F_9$ Col×Nd-1 plants was provided by E. Holub and J. Vicente (University of Warwick, UK). Plants were grouped into different categories according to the interaction phenotypes recorded. The Chi-square ($X^2$) test (McDonald, 2008) was performed to determine the goodness-of-fit of the data to the segregation ratios expected assuming different genetic models (*e.g.*, 3 resistant : 1 susceptible or 15 resistant : 1 susceptible, in case of one or two genes respectively, controlling dominant resistance). The hypothesis that a certain gene model fits the data was not rejected for $X^2$ probability greater than 0.05. This analysis was performed using Excel 2011 (Microsoft).

### 3.2.11 Statistical analysis for interval mapping

Chromosome locations of loci controlling resistance in *A. thaliana* to the *Xcr* strain HRI8305 were identified using MAGIC lines, and based on interaction phenotypes observed in two plants of each line following spray inoculation with this strain and the genotype data for 1260 single nucleotide polymorphisms determined by Kover *et al.* (2009). The maximum phenotype scores observed in two plants of each of the 353 MAGIC lines tested (in two experiment repeats) were used for the analysis. The mapping analysis was performed using the R software package HAPPY as described by Kover *et al.* (2009) (http://mus.well.ox.ac.uk/magic). A genome scan was performed using the function 'scan.phenotypes' to find significant genetic loci associated with the phenotypic trait under study (*i.e.* loci where the logP of genetic association is genome-wide significant with permutation p-value < 0.001). The genome-wide thresholds for statistical significance were determined with 10,000 permutations.

The map interval of the first targeted locus controlling resistance in Columbia was determined using a Col×Nd-1 recombinant inbred mapping population (Holub & Beynon, 1997). Phenotype data for 84 lines inoculated with *Xcr* race 2 strain HRI8305 was provided by E. Holub and J. Vicente (University of Warwick, UK). The maximum interaction phenotype scores observed in two to four plants of each of the 84 lines tested (in two experiment repeats) were used for the analysis. Initially, a genetic map was generated using genotype data for 103 molecular markers distributed across the genome of *A. thaliana* (data obtained from previous investigations including Deslandes *et al.* (1998), Werner *et al.* (2005) and E. Holub, pers. comm.). The genetic map was calculated using the Haldane map function (Haldane, 1919) implemented in R/qtl software (Broman *et al.*, 2003). The Microsoft Excel macro MapDraw 2.1 (Liu & M., 2003) was used for drawing the genetic map (presented in Appendix 7). Phenotype data was then used to define a map interval of the candidate locus using R/qtl software (Broman *et al.*, 2003) following the method described by Walley *et al.* (2012). Conditional genotype probabilities were estimated at maximum distance 1 cM and genotyping error rate 0.001 using the function 'calc.genoprob'. The genome scan was performed using the function 'scan.one' and the expectation-maximization 'EM' algorithm (Dempster *et al.*, 1977). The genome-wide LOD (Logarithm of the Odds Ratio) significance

threshold (p-value < 0.001) was determined by a permutation test with 10,000 iterations. LOD is a score that measures the strength of evidence for the presence of a locus associated with the phenotypic trait under study (Broman & Sen, 2009).

### 3.2.12 Clones for plant transformation

Different clones of genomic DNA from the resistant accession Col-0 were used for transformation of *A. thaliana* lines susceptible to *Xcr* race 2 strain HRI8305 and include: three large clones (ca. 90 kb each) designated JAtY57K17, JAtY64J11 and JAtY62J13, which cover a region on chromosome 3 that was predicted to contain a locus for resistance to *Xcr* race 2; and clones of the candidate gene identified in the present study to control resistance to *Xcr* race 2 (At3g57710) and the neighbouring gene At3g57700 (used as a negative control).

The JAtY clones were selected by using the Gbrowse tool (TAIR website, https://www.arabidopsis.org) and were purchased from Genome Enterprise Limited (Norwich, UK). They span the chromosome 3 as follows: clone JAtY57K17 from 21,252,931 to 21,341,148 bp, clone JAtY64J11 from 21,327,471 to 21,417,792 bp, and clone JAtY62J13 from 21,401,699 to 21,489,492 bp. For plasmid extraction from *Escherichia coli* cultures carrying JAtY clones, bacteria were initially grown in LB agar (Appendix 2) with 50 µg/ml kanamycin for 12 h at 37 °C followed by growth in LB broth with 50 µg/ml kanamycin and 0.7 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 5 h at 37 °C with agitation. Plasmid extraction was carried out using the Plasmid Mini kit (Qiagen) and following the manufacturer's instructions. Plasmids were then introduced into *A. tumefaciens* strain GV3101 (pMP90) by electroporation using a Bio-Rad electroporator and following the method described by Weigel & Glazebrook (2002).

The presence of each clone in transformed *A. tumefaciens* bacteria was confirmed by PCR amplification using the primers listed in Table 13 (VP primers). Primers were designed to amplify fragments of genes from both ends of each clone as well as from the mid region, and therefore detect the full length of the insert. Target genes in each clone were selected by using the GBrowse tool (TAIR website, https://www.arabidopsis.org; data source TAIR10) and specific primers were designed using the Primer-BLAST online tool (Ye *et al.*, 2012). DNA was extracted

by freezing and thawing of bacterial suspensions ($A_{640nm}$ 0.2) from each of three individual colonies of each transformed *A. tumefaciens* culture. PCR amplifications were carried out using Biomix (Bioline) as described in Section 3.2.6 with 1 µl of bacterial suspension.

**Table 13**. List of primers used to amplify genomic fragments of *Arabidopsis thaliana* accession Col-0 containing the genes At3g57700 and At3g57710 as well as fragments of the JAtY clones 57K17, 64J11 and 62J13

| Primer name | Gene (position, Mb[a]) | JAtY clone | Sequence (5' – 3') |
|---|---|---|---|
| pGN710-F | At3g57710 (21.385) | 64J11 | CGATTTCATCAAACAAATACTGTTG |
| pGN710-R | | | GAGAAACATATATGACCATGCAACC |
| pGN700-F | At3g57700 (21.385) | 64J11 | TCAGCTACTACTTGCACTAAGC |
| pGN700-R | | | CTAGCTAGAGATCCGCAAGGAAC |
| VP30-F | At3g57430 (21.256) | 57K17 | GCAGGTGTTGTTCCTGCTTG |
| VP30-R | | | TTGGTTCAACCCCGTAGTCG |
| VP20-F | At3g57530 (21.297) | 57K17 | TGCAGAATTGCAATGGCCTCGT |
| VP20-R | | | ACCTTGGGCCATGGGTCCCT |
| VP29-F | At3g57560 (21.311) | 57K17 | AATTCGCCGCCGCTTCCTCC |
| VP29-R | | | GGCCGCAAGCTCTCCAGCAA |
| VP31-F | AT3G57620 (21.338) | 57K17 | TCTTCTGGTGCGGTTCTTCC |
| VP31-R | | | AATCGGGTCACGTGCTTTCT |
| VP32-F | At3g57590 (21.330) | 64J11 | CTGTTTGCTGCCGAACGAAA |
| VP32-R | | | TCGGCATCCTCTAGAACCCA |
| VP28-F | At3g57660 (21.354) | 64J11 | GGTCACCCGCCTTTCACCGT |
| VP28-R | | | CCCCCAGCTCTAGGGGACCA |
| VP33-F | At3g57800 (21.408) | 64J11, 62J13 | GGGATTTCTTCAGACCGCCA |
| VP33-R | | | AAGCAAAGCCGTGAACGAAC |
| VP34-F | At3g57785 (21.405) | 62J13 | AAGCCATTGATTGTCGCAGC |
| VP34-R | | | AAGAGCTTTCTGGTGAGCCC |
| VP26-F | At3g57830 (21.420) | 62J13 | GCGCCATTGGATGATGCGGC |
| VP26-R | | | AGCCCGAGCCTCAGGTGCAA |
| VP35-F | At3g58030 (21.484) | 62J13 | TGTTCAGCGCAAGTGAAAGC |
| VP35-R | | | TGTCCTATGCCGAGTCCTGA |

[a] Physical positions in the AGI map of chromosome 3 of *A. thaliana* Columbia (AGI, 2000; TAIR website, https://www.arabidopsis.org; data source TAIR10).

Cloning of genes At3g57700 and At3g57710 was carried out by V. Çevik (University of Warwick, UK). Three genomic fragments containing either the gene At3g57710 or gene At3g57700 or both genes together, were amplified by PCR from genomic DNA of Col-0. The genomic fragment containing the protein coding region of At3g57710 (1056 bp) plus 486 bp upstream of the coding region (5' end) and 308 bp downstream of the coding region (3' end), was amplified using the primers pGN710-F and pGN710-R (Table 13). The genomic fragment containing the protein coding region of At3g57700 (1023 bp) plus 482 bp upstream of the coding region (5' end) and 301 bp downstream of the coding region (3' end), was amplified using the primers pGN700-F and pGN700-R (Table 13). The genomic fragment containing

the protein coding region of both genes (At3g57710 and At3g57700) plus 482 bp upstream of the coding region of At3g57700 (5' end) and 486 bp upstream of the coding region of At3g57710 (5' end), was amplified using the primers pGN700-F and pGN710-F (Table 13). The PCR products were cloned into the pCR8/GW/TOPO vector using the pCR8/GW/TOPO TA cloning kit (Invitrogen) and following the manufacturer's instructions, and the clones were sequenced and verified. The resulting entry vectors were then recombined with the pBGW binary vector (Karimi *et al.*, 2002) using Gateway LR Clonase II enzyme mix (Invitrogen) and following the manufacturer's instruction, and then introduced into *A. tumefaciens* strain GV3101 (pMP90).

### 3.2.13 *Arabidopsis thaliana* transformation

The *A. thaliana* lines selected for transformation were: Nd-1 which was used for transformation with JAtY clones and clones of genes At3g57710 and At3g57700; and Col-0 T-DNA insertion mutant SALK204228C and the $F_9$ Col-0xNd-1 recombinant inbred 3790 which were used for transformation with clones of genes At3g57710 and At3g57700. The floral-dip method described by Bent (2006) was used to transform *A. thaliana* with a suspension of *A. tumefaciens* bacteria carrying each of the clones of interest. Transformed *A. tumefaciens* were grown in LB broth with gentamicin 25 µg/ml, rifampicin 50 µg/ml and kanamycin 50 µg/ml (for those transformed with JAtY clones) or spectinomycin 100 µg/ml (for remaining clones), at 28 °C for 48 h with agitation. Bacterial suspensions were then centrifuged and re-suspended in 5% (w/v) sucrose (or glucose) solution with 0.05% (v/v) Silwet L-77. Glucose instead of sucrose was used for transformation with JAtY clones to increase transformation efficiency with these large clones according to the protocol developed by J. Alonso's group at the North Carolina State University (http://www4.ncsu.edu/~jmalonso/Alonso-Stepanova_JAtY.html). Flowers from 7 to 8-week old plants were immersed into bacterial suspensions for approximately 10 s and covered with a plastic bag for 12 to 24 h.

Selection of transgenic plants was performed by herbicide resistance selection. Primary transgenic seeds ($T_1$ seeds) were germinated in a compost mix regularly watered with sterile Murashige & Skoog solution (Duchefa Biochemie) with 0.1%

(v/v) Basta herbicide (glufosinate ammonium; Bayer CropScience). $T_1$ plants which are hemizygous for the clone of interest (Bent, 2006), were self-pollinated to select $T_2$ progenies that showed 3:1 segregation ratio of live:dead seedlings for Basta resistance indicating presence of single copy/locus insert. Plants from selected $T_2$ progenies were then self-pollinated for selection of $T_3$ progenies that exhibited 100% resistance to Basta and are likely homozygous for the insert. $T_3$ plants were obtained for JAtY clones whereas only $T_1$ plants were obtained for clones of genes At3g57710 and At3g57700.

### 3.2.14 Measurement of bacterial growth *in planta*

The growth *Xcr* strain HRI8305 was assayed on seven *A. thaliana* accessions, including: Col-0, Col-5, Nd-1, Oy-0, the $F_9$ Col-0×Nd-1 recombinant inbred line 3790, the homozygous Col-0 mutant SALK204228C and the homozygous transgenic line Nd-1::JAtY64J11 obtained in this study (as described in Section 3.2.13). Four-week-old plants were spray-inoculated following the method described in Chapter 2 (Section 2.2.5). All plants used for inoculation had at least four adult leaves. The density of viable bacteria in inoculated leaves was determined at five time points: immediately following spray inoculation, and at one, three, five and seven days after inoculation.

At each time point and for each *A. thaliana* accession, six plants were sampled (biological replicates). One disc (diameter 0.6 cm) was excised from each of the first four adult leaves emerged in each rosette, using a cork borer. Each set of four leaf discs was placed in a 2 ml microcentrifuge tube with 200 µl sterile 10 mM $MgCl_2$ solution and two tungsten carbide beads (3 mm). Leaf discs were ground twice for 30 s at 25 pulses/s using a mixer mill MM 300 (Retsch).

Samples were diluted in sterile 10 Mm $MgCl_2$. A volume of 300 µl sterile 10 mM $MgCl_2$ was initially added to each sample and then 10-fold serial dilutions ($10^{-1}$ to $10^{-6}$) of each sample were prepared in 96-well plates kept in a shaker platform (Titramax 100, Heidolph) to assure constant homogenization of the samples. Aliquots of 10 µl of each dilution were spotted in a square plate containing King's B medium (King *et al.*, 1954; Appendix 2). This process was repeated to inoculate four plates and obtain four bacterial counts for each dilution (technical replicates).

Plates were incubated at 28 °C and bacterial colonies were counted 24 to 32 hours after plating. The method described here is an adaptation of the method described by Katagiri *et al.* (2002).

The numbers of colony-forming units per square centimetre of leaf area (cfu/cm$^2$) were estimated for each sample and transformed into a logarithmic scale. Differences between the log(cfu/cm$^2$) means obtained for each accession and time point were assessed by analysis of variance using GenStat version 15 (VSN International). The Least Significant Difference (LSD) between means (*i.e.* the smallest difference between means that would result in statistical significance) was calculated at significance level 0.05. The standard error of the mean was calculated as the square root of, the residual mean square divided by the number of observations on each mean (24 observations; 6 biological replicates × 4 technical replicates). This analysis was performed with support from A. Mead and J. Jones (University of Warwick, UK).

### 3.2.15 Sequence analysis of the *Arabidopsis thaliana* gene At3g57710

The sequences of the gene At3g57710 and intergenic region between this gene and AT3G57720, of 68 *A. thaliana* accessions were retrieved from the *Arabidopsis* 1001 genomes database (http://signal.salk.edu/atg1001). The selected accessions included those identified in the present study as resistant or susceptible to the *Xcr* race 2 strain HRI8305 (Sections 2.3.3 and 2.3.5, Chapter 2) and that had the genome sequence publicly available at the time of this analysis (http://signal.salk.edu/atg1001). The DNA sequences were aligned and compared using Geneious software 6 (Kearse *et al*., 2012).

## 3.3 RESULTS

### 3.3.1 Mapping of resistance to *Xanthomonas campestris* pv. *raphani* race 2 (strain HRI8305) in MAGIC lines of *Arabidopsis thaliana*

A total of 353 MAGIC lines were inoculated with *Xcr* race 2 strain HRI8305. For each line, two plants were tested and the maximum phenotype score observed among those two plants was recorded (results are presented in Appendix 4). The mapping analysis using this dataset identified two significant loci (genome wide permutation p-value < 0.001) for **R**esistance to ***Xcr*** (*RXCR*) that are between positions 20,256,936 and 23,291,586 bp on chromosome 3 (locus designated as *RXCR1* located within 3.03 Mb), and between positions 16,429,063 and 22,697,401 bp on chromosome 5 (locus designated as *RXCR2* located within 6.27 Mb) (Table 14, Figure 9). The most significant single nucleotide polymorphism (SNP) identified in each of these map intervals were at the physical positions, 21,116,640 bp for the *RXCR1* locus (chromosome 3; SNP MN3-21127619) and 19,684,471 bp for the *RXCR2* locus (chromosome 5; SNP MASC01039) (Table 14, Figure 9).

**Table 14**. Significant loci detected by mapping resistance to *Xanthomonas campestris* pv. *raphani* race 2 (strain HRI8305) in *Arabidopsis thaliana* using 353 MAGIC lines

| Chr | Peak (bp)[a] | SNP marker | Predicted map interval (bp)[a] | logP[b] | Genome-wide P[b] |
|---|---|---|---|---|---|
| 3 | 21,116,640 | MN3-21127619 | 20,256,936 - 23,291,586 | 7.54 | 0 |
| 5 | 19,684,471 | MASC01039 | 16,429,063 - 22,697,401 | 17.22 | 0 |

Abbreviations: Chr, chromosome; MAGIC lines, Multi-parent Advanced Generation Inter-Cross inbred lines (Kover *et al.*, 2009); bp, base pair; P, probability; SNP, single nucleotide polymorphism.

[a] Physical positions are based on the reference genome of *A. thaliana* Columbia (data source TAIR10; https://www.arabidopsis.org/).

[b] Statistical significance of each locus is indicated by logP and genome-wide permutation p-value.

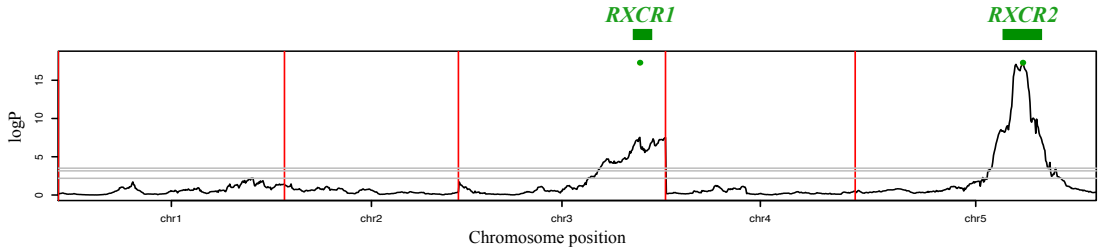
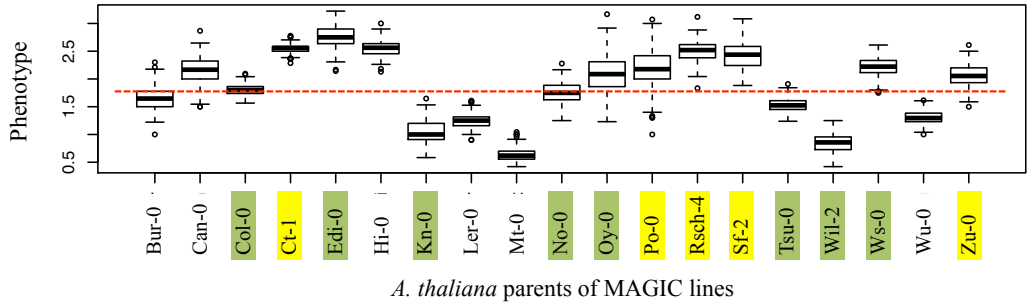



**Figure 9**. Identification of two loci (designated *RXCR1* and *RXCR2*) that control resistance to *Xanthomonas campestris* pv. *raphani* (race 2, strain HRI8305) relative to segregation of SNP marker variation in a Multi-parent Advanced Generation Inter-Cross (MAGIC) inbred population of *Arabidopsis thaliana*. The genome scans were obtained using the assessment of the interaction phenotypes in 353 MAGIC inbred lines. For each line, two plants were tested in two experiment repeats and the maximum phenotype score observed was used for the analysis. Vertical red lines indicate the chromosome boundaries; grey horizontal lines indicate the permutation derived genome-wide thresholds at 50%, 90% and 95%; green dots indicate the position of the most significant SNP marker (p-value < 0.001) and green bars indicate the predicted map intervals.

Estimated contributions of each of the 19 MAGIC parent accessions to heritable phenotypic variation among the MAGIC inbreds at the most significant SNPs that predict the location of *RXCR1* and *RXCR2* loci, are summarized in Figure 10. The highest phenotypic level on the Y-axis corresponds to susceptibility and the lowest level corresponds to resistance. These plots may give an indication of which parents have a resistance allele at each locus. For example, Columbia (Col-0) was used in follow-up experiments to identify a resistance gene at the *RXCR1* map interval (described below). Five susceptible parents (Ct-1, Po-0, Rsch-4, Sf-2 and Zu-0 as confirmed in Chapter 2) all have a higher phenotypic level than Col-0 at the SNP position on chromosome 3 (Figure 10a). Eight parents have the same or lower phenotypic level than Col-0 at the same position. Four of these parents (Kn-0, No-0, Tsu-0 and Wil-2) were confirmed as being resistant in Chapter 2 and may therefore have a *RXCR1* resistance allele. Interestingly, three resistant parents (Oy-0, Edi-0, and Ws-0) have a higher phenotypic level than Col-0 at this locus. However, these have a phenotypic level near zero along with Wil-2 at the SNP position on chromosome 5 (Figure 10b), suggesting that these parents may share a *RXCR2* resistance allele that is not present in Col-0.

**a** **Chromosome 3, 21,116,640 bp (locus *RXCR1*)**




**b** **Chromosome 5, 19,684,471 bp (locus *RXCR2*)**

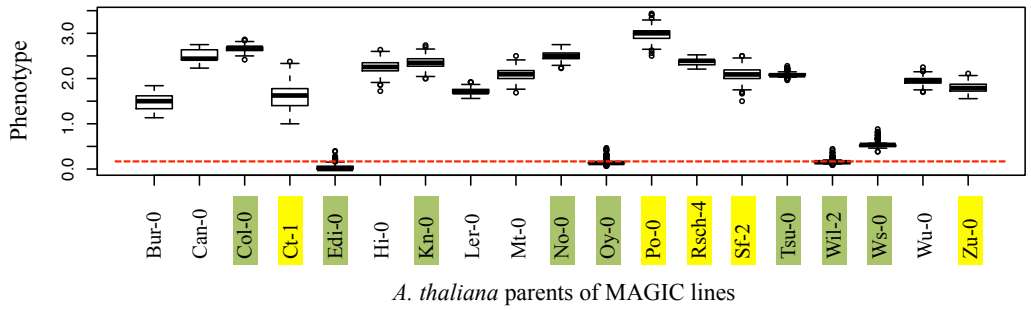



**Figure 10**. Estimated contributions of 19 *Arabidopsis thaliana* accessions to phenotypic variation, at the most significant molecular markers for predicting the location of the two loci designated *RXCR1* (a) and *RXCR2* (b) that control resistance to *Xanthomonas campestris* pv. *raphani* (race 2, strain HRI8305). The loci were genetically mapped using 353 inbreds from a Multi-parent Advanced Generation Inter-Cross (MAGIC) population that was derived from the 19 accessions (see Figure 9). The estimates were determined using the R package HAPPY (Kover *et al.*, 2009). The Y-axis indicates the range of phenotypes from no symptoms (0 or resistance) to the highest severity of symptoms (susceptibility). Resistant and susceptible parents are indicated on the X-axis with green or yellow highlighting, respectively according to the results presented in Table 6 (Chapter 2); no highlighting indicates an inconclusive phenotype. The horizontal red-dashed line indicate the phenotypic level of the resistant accession that was used in subsequent experiments to confirm a map interval for *RXCR1* (Col-0 in plot a) and *RXCR2* (Oy-0 in plot b).

To test the hypothesis that Col-0 and Oy-0 are representative parents that have a resistance allele at either *RXCR1* or *RXCR2* respectively, six $F_8$ recombinant inbreds derived from a cross between these accessions were spray-inoculated with strain HRI8305. These inbreds were chosen as representative lines that lack Col-0 marker alleles across the *RXCR1* map interval, but differ in having either Col-0 or Oy-0 marker alleles across the *RXCR2* map interval (Appendix 8), based on publicly available genotype data for 85 molecular markers (Simon *et al.*, 2008; http://publiclines.versailles.inra.fr). Two inbreds (107 and 127) exhibited

phenotypes similar to the susceptible control Nd-1, indicating that Col-0 and Oy-0 do not share a resistance allele at the same locus (*RXCR1*), and that Oy-0 may therefore have a resistance allele at the *RXCR2* locus.

### 3.3.2 Inheritance and coarse-scale mapping of *RXCR1* and *RXCR2*

The *A. thaliana* accessions Columbia and Oystese were used in conventional two-parent crosses to the susceptible accession Nd-1 to further investigate resistance to *Xcr* race 2 (strain HRI8305) at the two major loci identified above in the MAGIC analysis (*RXCR1* and *RXCR2*).

Spray inoculation with the *Xcr* strain HRI8305 of ten $F_1$ plants derived from reciprocal crosses of Col-5 with Nd-1 resulted in absence of typical leaf spots (interaction phenotype, IP, 0-1) or limited development of leaf spots (IP 2), similar to the phenotypes observed in plants of the resistant control (Col-5) (Figure 11, Table 15). This suggests that the Columbia allele of *RXCR1* confers a dominant phenotype, and that there is no maternal effect in the expression of resistance. The $F_2$ segregation of resistance was investigated using 1020 plants derived from a Col-5×Nd-1 cross. Nearly 25% of the plants (246) were fully susceptible exhibiting the phenotype of the susceptible parent Nd-1 (IP 3-5); whereas the remaining plants (774) showed full resistance (IP 0-1) or limited development of leaf spots (IP 2) as observed in the resistant parent Col-5 (Table 15). These data fit a ratio of 3 resistant : 1 susceptible ($X^2$ probability 0.505, Table 15) assuming that one gene is controlling resistance, whereas other genetic models assuming two genes (*e.g.* 15:1 or 9:7) or three genes (*e.g.* 57:7) controlling resistance were not consistent with the data observed. The $F_2$ data therefore supports the prediction that an allele in Columbia confers dominant resistance to *Xcr* race 2 (HRI8305) at the *RXCR1* locus. In addition, segregation of resistance in 84 $F_9$ Col×Nd-1 recombinant inbreds, which included 39 lines that scored as resistant (IP 0-2) and 45 lines that scored as susceptible (IP 3-5) (phenotype data provided by E. Holub and J. Vicente), also had a good fit for a ratio of 1 resistant : 1 susceptible ($X^2$ probability 0.513, Table 15) that is expected for a single gene conferring resistance to *Xcr* race 2.

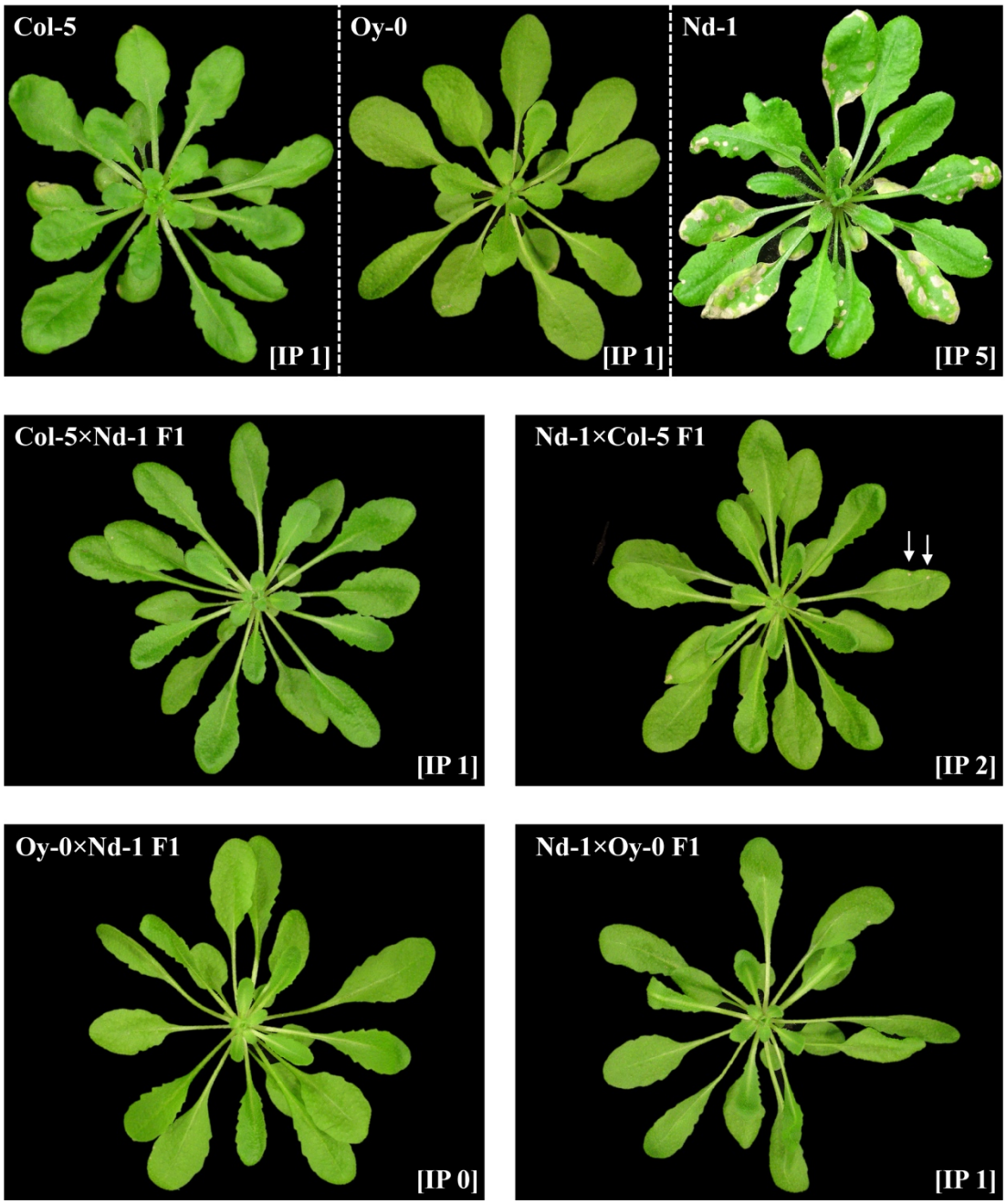



**Figure 11**. Interaction phenotypes following spray inoculation with *Xanthomonas campestris* pv. *raphani* race 2 (strain HRI8305) of three standard *Arabidopsis thaliana* accessions (Columbia, Col-5; Oystese, Oy-0; and Niederzenz, Nd-1) and $F_1$ plants that were derived from reciprocal crosses of Nd-1 (susceptible) with Col-5 or Oy-0 (resistant). Photographs were taken seven days after spray inoculation. White arrows indicate presence of small leaf spots in one $F_1$ plant. The interaction phenotype (IP) scores are indicated within brackets and were assessed based on a six-point phenotype scale (see Chapter 2, Figure 4).

**Table 15**. Inheritance of resistance to *Xanthomonas campestris* pv. *raphani* (strain HRI8305) in *Arabidopsis thaliana* $F_1$, $F_2$ and $F_9$ plants derived from crosses of accessions Columbia (Col-5 or Col-0) and Niederzenz (Nd-1)

| Line (cross)[a] | Number of plants[b] | | | | Ratio | | $X^2$ |
|---|---|---|---|---|---|---|---|
| | Total | R[0-1] | R[2] | S[3-5] | Observed | Predicted | probability |
| Col-5 | 54 | 52 | 2 | | | | |
| Nd-1 | 54 | | | 54 | | | |
| **$F_1$** | | | | | | | |
| (Col-5×Nd-1) | 10 | 8 | 2 | | | | |
| (Nd-1×Col-5) | 10 | 9 | 1 | | | | |
| **$F_2$** | | | | | | | |
| (Col-5×Nd-1) | 1020 | 473 | 301 | 246 | 774:246 (R:S) | 3:1 | 0.515 |
| **$F_9$** | | | | | | | |
| (Col×Nd-1) | 84 | 5 | 34 | 45 | 39:45 (R:S) | 1:1 | 0.513 |

[a] $F_1$, plants derived from reciprocal parental crosses with *A. thaliana* accessions Col-5 and Nd-1; $F_2$, plants derived from self-pollination of one $F_1$ Col-5×Nd-1 plant; $F_9$, recombinant inbred lines derived from crosses between Columbia (Col-0 or Col-5) and Nd-1.

[b] Plants were spray-inoculated with the *X. campestris* pv. *raphani* strain HRI8305 and scored for symptoms 10 days after inoculation. Interaction phenotypes (IP) were scored using a six-point scale (see Chapter 2, Figure 4). Plants were grouped into two different classes: resistant (R) if showing IP 0 to 2 (IP 0 to 1 indicate absence of leaf spots and IP 2 indicate limited development of leaf spots); and susceptible (S) if showing IP 3 to 5 (typical leaf spot symptoms). Interaction phenotypes of each group are indicated within brackets. $F_9$ recombinant inbred lines were grouped according to the maximum IP score observed in four plants of each line tested in two experiment repeats (phenotype data provided by E. Holub and J. Vicente, University of Warwick, UK).

Statistical mapping analysis of available genotype data for 103 molecular markers and phenotype scores of interactions between 84 $F_9$ Col×Nd-1 inbred lines and *Xcr* strain HRI8305, confirmed a single *RXCR1* locus at the bottom arm of chromosome 3 in the accession Columbia (Figure 12). A LOD score peak of 16.54 was observed at 97.0 cM, with gen7250 and nga112 (95.7 and 101.4 cM, respectively) as the nearest markers on either side of the peak delimiting a physical map interval of 1.87 Mb in the reference genome of *A. thaliana* Columbia (between 21,309,228 and 23,179,526 bp). This locus appeared to be closer to gen7250 because a drop of 2 LOD (the interval in which the LOD score is within 2 units of its maximum; Broman & Sen, 2009) suggests a locus at 95.7 to 100 cM, which is nearest the position of gen7250 in the genetic map (as shown in Appendix 7). This marker is also closer to the SNP (MN3-21127619 at 21,116,640 bp) that was identified as the most significant linked marker in the analysis of MAGIC data described above (Table 15).

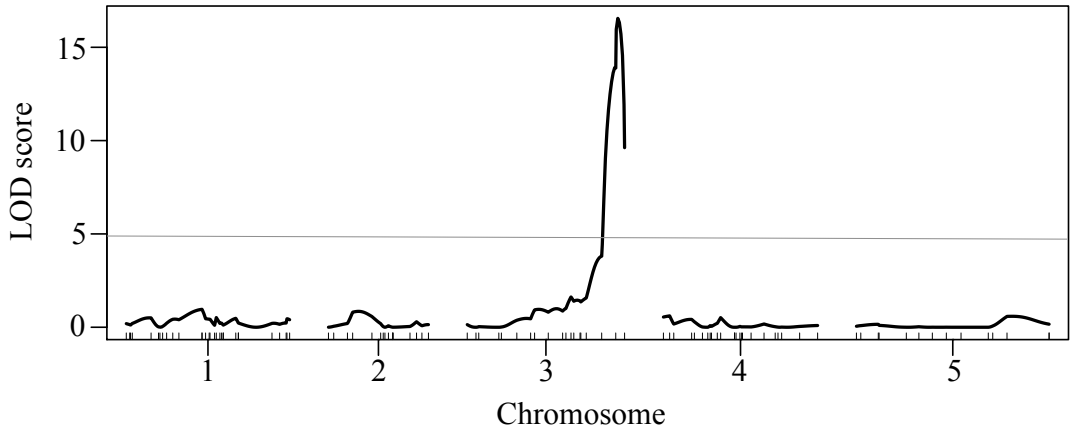



**Figure 12**. Identification of a single locus on chromosome 3 of *Arabidopsis thaliana* accession Columbia that confers resistance to *Xanthomonas campestris* pv. *raphani* race 2 strain HRI8305. The locus was mapped using $F_9$ Col×Nd-1 inbreds relative to 103 molecular markers. The grey horizontal line indicates the genome-wide significance threshold (p-value < 0.001) estimated with 10,000 permutations.

To investigate resistance in Oy-0 to the *Xcr* strain HRI8305, $F_1$ plants derived from reciprocal crosses with Nd-1 were inoculated and showed no development of typical leaf spots (IP 0-1) similar to the phenotypes observed in the resistant control plants of Oy-0 (Figure 11, Table 16). This indicates that a resistance allele in Oy-0 also confers a dominant phenotype with no maternal effect. Segregation of this resistance in 158 $F_2$ plants derived from a Nd-1×Oy-0 cross included: 59 plants that were fully susceptible (IP 3-5) as observed in susceptible control plants of Nd-1, 87 plants that exhibited absence of leaf spots (IP 0-1) as observed in the resistant parent Oy-0, and 12 plants that showed limited development of leaf spots (IP 2) (Table 16). These data could be explained with a genetic model of two complementary genes controlling resistance (*e.g.* 9 resistant : 7 susceptible) if the class IP 2 is combined with the resistant class IP 0-1 ($X^2$ probability 0.104) (Table 16). Other genetic models for the control of resistance such as one-gene model (*e.g.* 3:1 or 1:2:1), two-gene model (*e.g.* 15:1) or a three-gene model (*e.g.* 57:7) were not consistent with the data observed. In 98 $F_9$ recombinant inbreds derived from a Nd-1×Oy-0 cross, 43 plants that were fully resistant or showed only limited development of spots (IP 0-2), and 55 plants that were fully susceptible (IP 3-5). These data fit an expected recombinant inbred ratio of 1 resistant : 1 susceptible ($X^2$ probability 0.225) (Table 16) for the presence of a single locus controlling resistance to *Xcr* race 2 in Oy-0.

Using the $F_9$ Nd-1×Oy-0 recombinant inbreds phenotyped in this study, the presence of the *RXCR2* locus for resistance in Oy-0 was confirmed on chromosome 5 in a similar region (6.9 Mb interval, 15.9 to 22.8 Mb) to the one predicted in the mapping analysis using MAGIC lines (6.27 Mb interval, 16.43 to 22.7 Mb). Also, no additional resistance gene was mapped to the *RXCR1* locus. This work was performed by U. Sonawala as part of her MSc research project (Sonawala, 2013) that I co-supervised with E. Holub.

**Table 16**. Inheritance of resistance to *Xanthomonas campestris* pv. *raphani* (strain HRI8305) in *Arabidopsis thaliana* $F_1$, $F_2$ and $F_9$ plants derived from crosses of accessions Niederzenz (Nd-1) and Oystese (Oy-0)

| Line (cross)[a] | Number of plants[b] | | | | Ratio | | $X^2$ |
|---|---|---|---|---|---|---|---|
| | Total | R[0-1] | R[2] | S[3-5] | Observed | Predicted | probability |
| Oy-0 | 30 | 10 | | | | | |
| Nd-1 | 28 | | | 28 | | | |
| **$F_1$** | | | | | | | |
| (Nd-1×Oy-0) | 10 | 10 | | | | | |
| (Oy-0×Nd-1) | 10 | 10 | | | | | |
| **$F_2$** | | | | | | | |
| (Nd-1×Oy-0) | 158 | 87 | 12 | 59 | 99:59 (R:S) | 9:7 | 0.104 |
| **$F_9$** | | | | | | | |
| (Nd-1×Oy-0) | 98 | 40 | 3 | 55 | 43:55 (R:S) | 1:1 | 0.225 |

[a] $F_1$, plants derived from reciprocal parental crosses with *A. thaliana* accessions Oy-0 and Nd-1; $F_2$, plants derived from self-pollination of one $F_1$ Nd-1×Oy-0 plant; $F_9$, recombinant inbred lines derived from a Nd-1×Oy-0 cross.

[b] Plants were spray-inoculated with the *X. campestris* pv. *raphani* strain HRI8305 and scored for symptoms 10 days after inoculation. Interaction phenotypes (IPs) were scored using a six-point scale (see Chapter 2, Figure 4). Plants were grouped into two different classes: resistant (R) if showing IP 0 to 2 (IP 0 to 1 indicate absence of leaf spots and IP 2 indicate limited development of leaf spots); susceptible (S) if showing IP 3 to 5 (typical leaf spot symptoms). IP scores are indicated within brackets; $F_9$ recombinant inbred lines were grouped according to the maximum IP score observed in three plants of each line tested in one experiment.

### 3.3.3 Fine-mapping of *RXCR1*

An effort to reduce the mapping interval of *RXCR1* was initiated using eight lines from the $F_9$ Col×Nd-1 mapping population that were recombinant within the 1.87 Mb interval delimited by the molecular markers *gen7250* and *nga112* (Table 17). The eight *A. thaliana* inbred lines were initially genotyped using the molecular markers m57530, m58310, m58350, m58470, m59070, m59890, m60940, m62080

(developed in this study within the target interval as described in Section 3.2.8). The use of these marker data in combination with the interaction phenotypes determined by plant inoculation with the *Xcr* strain HRI8305 allowed the identification of a narrower interval of 286.25 kb, delimited by the markers m57530 and m58310 as shown in Table 17. Plant lines 3790 and 3893 were particularly informative to define this interval. The line 3790 is susceptible to HRI8305 and has the Columbia allele (resistant parent) at the markers m57530/gen7250 and the Niederzenz allele (susceptible parent) in the downstream markers. This suggests that *RXCR1* is downstream of these markers. The line 3893 is resistant to HRI8305 and has the Columbia allele at the marker m58310 and the Niederzenz allele in the upstream markers, which suggests that *RXCR1* is upstream of this marker.

To define a narrower interval, additional plants that were recombinant within the interval of 286.25 kb were selected from 79 previously untested $F_9$ Col×Nd-1 inbred lines (listed in Appendix 5 together with the original eight recombinant lines) and 1020 $F_2$ Col×Nd-1 plants. For this purpose, all plants were genotyped with the flanking interval markers m57530 and m58310 (Table 17, Appendix 5). Four $F_9$ and 13 $F_2$ lines were identified as recombinants within the 286.25 kb map interval, and were tested for resistance to HRI8305. These plants as well as the eight original recombinants were genotyped using four additional markers (m57660, m57830, m58090 and m58230) within the 286.25 kb interval. $F_3$ families (derived from each of the 13 $F_2$ recombinant lines) were also tested for segregation of resistance to enable prediction of the $F_2$ genotype at the *RXCR1* locus (Table 17). If susceptible plants were identified within a given $F_3$ family, then the $F_2$ parent was predicted to have inherited at least one *RXCR1* allele from the susceptible parent Nd-1. Prediction for the second allele was decided based on the ratio of resistant (IP 0-2) and susceptible (IP 3-5) plants observed in the $F_3$ family as follows: a higher proportion of resistant plants was interpreted as a second allele from the resistant parent Col-5 (the $F_2$ parent was heterozygous for the *RXCR1* locus), whereas a higher proportion of susceptible plants was interpreted as a second allele from Nd-1 (the $F_2$ parent was homozygous for the *RXCR1* locus).

**Table 17**. Summary of key recombinants used to define a fine-map interval for the *RXCR1* locus in *Arabidopsis thaliana* conferring resistance to *Xanthomonas campestris* pv. *raphani* strain HRI8305. The experimental plant lines were derived from crosses between accessions Columbia (Col-5 or Col-0, resistant) and Niederzenz (Nd-1, susceptible)

| Plant line | IP[a] | $F_3$ ratio (R:S)[b] | Genotype at molecular marker [c] (position in Mb) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | m57530 (21.30) | gen7250 (21.31) | m57660 (21.36) | *RXCR1*[d] | m57830 (21.42) | m58090 (21.51) | m58230 (21.57) | m58310 (21.58) | m58350 (21.59) | m58470 (21.63) | m59070 (21.83) | m59890 (22.13) | m60940 (22.52) | m62080 (22.99) | nga112 (23.18) |
| **Parents** | | | | | | | | | | | | | | | | | |
| Col-5 | R[2] | | CC | CC | CC | CC | CC | CC | CC | CC | CC | CC | CC | CC | CC | CC | CC |
| Nd-1 | S[5] | | NN | NN | NN | NN | NN | NN | NN | NN | NN | NN | NN | NN | NN | NN | NN |
| **$F_9$ lines used to refine the 1.87 Mb interval to a smaller interval (286.25 Kb)** | | | | | | | | | | | | | | | | | |
| 3800 | R[1] | na | CC | CC | CC | CC | CC | CC | CC | CC | CC | CC | CC | CC | CC | CC | NN |
| 3819 | R[1] | na | CC | CC | CC | CC | CC | CC | CC | CC | CC | CC | NN | NN | NN | NN | NN |
| 3858 | R[1] | na | CC | CC | CC | CC | CC | CC | CC | CC | CC | CC | CC | CC | CC | CC | NN |
| 3790 | S[4] | na | CC | CC | NN | NN | na | NN | NN | NN | NN | NN | NN | NN | NN | NN | NN |
| 3860 | S[4] | na | NN | NN | NN | NN | NN | NN | NN | NN | NN | NN | NN | NN | NN | NN | CC |
| 3887 | S[5] | na | NN | NN | NN | NN | NN | NN | NN | NN | NN | NN | CC | CC | CC | CC | CC |
| 3892 | S[4] | na | NN | NN | NN | NN | NN | NN | NN | NN | NN | NN | NN | NN | NN | CC | CC |
| 3893 | S[3] | na | NN | NN | NN | NN | NN | NN | NN | CC | CC | CC | CC | CC | CC | CC | CC |
| **Additional $F_9$ recombinant lines that confirmed the interval delimited by markers m57530 and m58310 (286.25 Kb)** | | | | | | | | | | | | | | | | | |
| 8011b | R[1] | na | CC | na | CC | CC | CC | CC | CC | NN | na | na | na | na | na | na | na |
| 8044 | R[1] | na | NN | na | CC | CC | CC | CC | CC | CC | na | na | na | na | na | na | na |
| 8048 | R[2] | na | NN | na | CC | CC | CC | CC | CC | CC | na | na | na | na | na | na | na |
| 8011a | S[4] | na | CN | na | NN | NN | NN | NN | NN | NN | na | na | na | na | na | na | na |
| **Recombinant $F_2$ lines within the 286.25 Kb interval used to predict a smaller interval (62.31 Kb)** | | | | | | | | | | | | | | | | | |
| 268 | R[1] | 25:13 | CN | na | CN | CN | CN | NN | NN | NN | na | na | na | na | na | na | na |
| 289 | R[1] | 56:5 | CN | na | CN | CN | CN | NN | NN | NN | na | na | na | na | na | na | na |
| 170 | R[2] | 61:2 | CC | na | CC | CN | CN | CN | CN | CN | na | na | na | na | na | na | na |
| 215 | R[2] | 25:6 | CN | na | CN | CN | CN | CN | CC | CC | na | na | na | na | na | na | na |
| 257 | R[2] | 67:4 | CN | na | CN | CN | CN | CN | CC | CC | na | na | na | na | na | na | na |
| 288 | R[2] | 21:14 | CN | na | CN | CN | CN | CN | CC | CC | na | na | na | na | na | na | na |
| 458 | R[2] | 16:13 | CN | na | CN | CN | CN | CC | CC | CC | na | na | na | na | na | na | na |
| 501 | R[2] | 45:24 | CN | na | CN | CN | CC | CC | CC | CC | na | na | na | na | na | na | na |
| 551 | R[2] | 25:7 | CN | na | CN | CN | CN | CN | CN | CC | na | na | na | na | na | na | na |
| 674 | R[2] | 64:3 | CC | na | CN | CN | CN | CN | CN | CN | na | na | na | na | na | na | na |
| 589 | S[4] | 12:60 | NN | na | NN | NN | NN | CN | CN | CN | na | na | na | na | na | na | na |
| 881 | S[3] | 17:49 | CN | na | NN | NN | NN | NN | NN | NN | na | na | na | na | na | na | na |
| 811 | R[2] | 2:28 | NN | na | NN | NN | NN | NN | NN | CN | na | na | na | na | na | na | na |

Abbreviations: CC, homozygous for the Columbia allele (highlighted in green), NN, homozygous for the Niederzenz allele (highlighted in yellow) and CN, heterozygous (highlighted in grey); na, not available; R, resistant; S, susceptible.

[a] The interaction phenotype (IP) scores determined by spray inoculation with *Xcr* strain HRI8305, are indicated within brackets and are according to the six-point scale developed in this study (see Chapter 2, Figure 4). Scores correspond to the maximum score observed in: four plants tested in two experiment repeats for $F_9$ lines; one unique $F_2$ plant; and 81 Col-5 and 79 Nd-1 plants used as controls in the experiments. Plant lines showing IP scores 0 to 2 were considered resistant (R); and 3 to 5 were considered susceptible (S).

[b] Segregation ratios of phenotypes observed in the $F_3$ progeny derived from self-pollination of each of the $F_2$ recombinant plants, are indicated according to the same criteria described in [a]. For each family, a total number of 29 to 72 plants were tested.

[c] Marker positions delimiting the intervals determined by genotyping the *A. thaliana* $F_9$ and $F_2$ lines are indicated within black rectangular lines. Molecular markers delimiting the initial interval are highlighted in blue; the genotype data for these markers was determined by Werner *et al.* (2005). The genotype data for the remaining markers was determined in the present study.

[d] Predicted position and genotypes for the *RXCR1* locus.

In $F_9$ plants, no recombination events were detected in the genotypes of the four markers identified within the 286.25 kb interval (m57660, m57830, m58090 and m58230). Therefore, these data only allowed confirmation of the interval (Table 17). However, in the $F_2$ recombinants, the combined genotypes of these markers and genotypes predicted for the *RXCR1* locus (based on segregation ratios of resistance in the $F_3$ families) indicated that *RXCR1* is located within a narrower 62.31 kb interval delimited by markers m57660 and m57830 (Table 17). According to the TAIR database (https://www.arabidopsis.org/), this narrower interval spans a region containing 20 genes in the reference genome of Columbia (Figure 13).

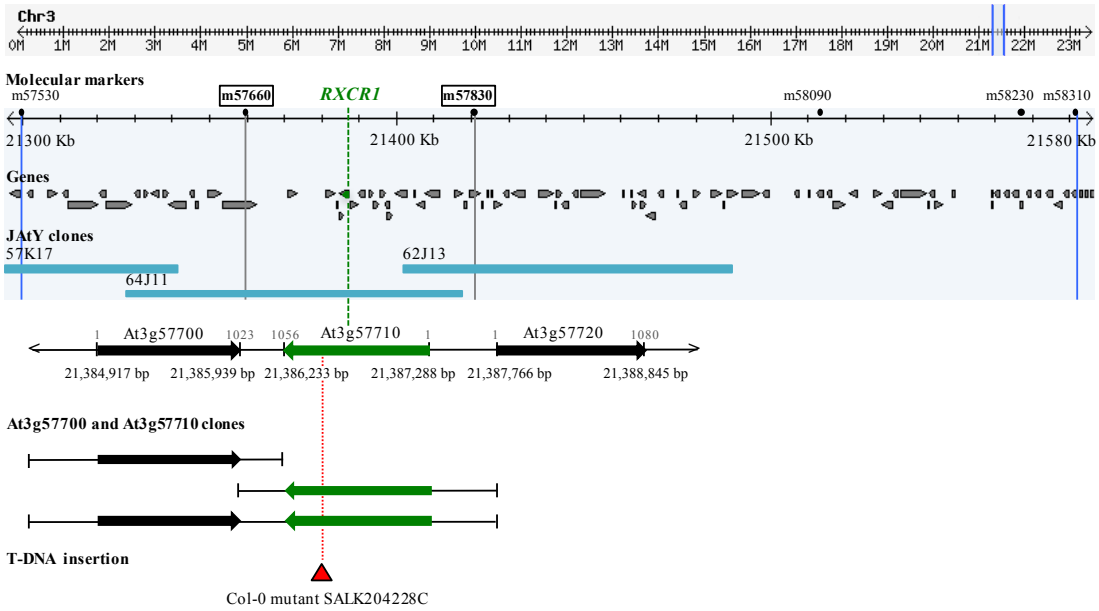



**Figure 13**. Physical map of the *RXCR1* locus within a 62.31 kb interval located on the bottom arm of chromosome 3 between molecular markers m57660 and 57830 as determined by genetic mapping. Large insert JAtY clones of genomic fragments from the resistance accession Columbia (Col-0) and spanning the map interval, are shown (drawn to scale) in the blue highlighted box (grey arrows represent genes). JAtY64J11 provided gain-of-function when transferred into susceptible accessions (see Figure 15). Three clones of genomic fragments from Col-0 containing genes At3g57700, At3g57710 and both genes, are indicated below, and only the bottom two provided gain-of-function. A red arrowhead indicates the position of a T-DNA insertion in the loss-of-function Col-0 mutant SALK204228C. A green arrowed line indicates the gene At3g57710 (confirmed as *RXCR1*), and black arrowed lines indicate the two neighbouring genes (At3g57700 and At3g57720).

### 3.3.4 Identification of *RXCR1* gene by loss- and gain-of-function experiments

A total of 19 Col-0 T-DNA insertion mutants (knockout mutants) were used for mutational analysis of 11 genes out of the 20 candidate genes that are located within the 62.31 kb map interval of *RXCR1* (mutants listed in Appendix 6). Following spray inoculation with the *Xcr* race 2 strain HRI8350, development of typical leaf spot symptoms (IP 3-5) similar to those observed in susceptible control plants (Nd-1), was only observed in plants of the knockout mutant line SALK204228C (Figure 14) containing an insert in the gene At3g57710 (Figure 13). Plants of the remaining 18 mutants tested showed no altered phenotype in comparison to the wild type Col-0 control plants (IP 0-2). A summary of the maximum and minimum scores for interaction phenotypes observed in plants of each mutant line tested are presented in Appendix 6.

PCR amplifications using a primer specific for the T-DNA insert (LBb1.3) and a primer specific for the gene At3g57710 (RP), produced a single fragment of the expected size (*ca.* 600 bp) from all eight phenotyped mutant plants, thus confirming the location of the insert in this gene. PCR amplifications using the genomic primers LP and RP specific for the flanking sequences on either side of the insert location, produced the expected fragment from a Col-0 wild type plant, but no fragment was amplified from the T-DNA mutant plants as expected for homozygous lines. According to the Columbia genome sequence annotated in the TAIR website (https://www.arabidopsis.org), At3g57710 lacks introns and is composed instead of a single protein coding sequence of 1056 bp.

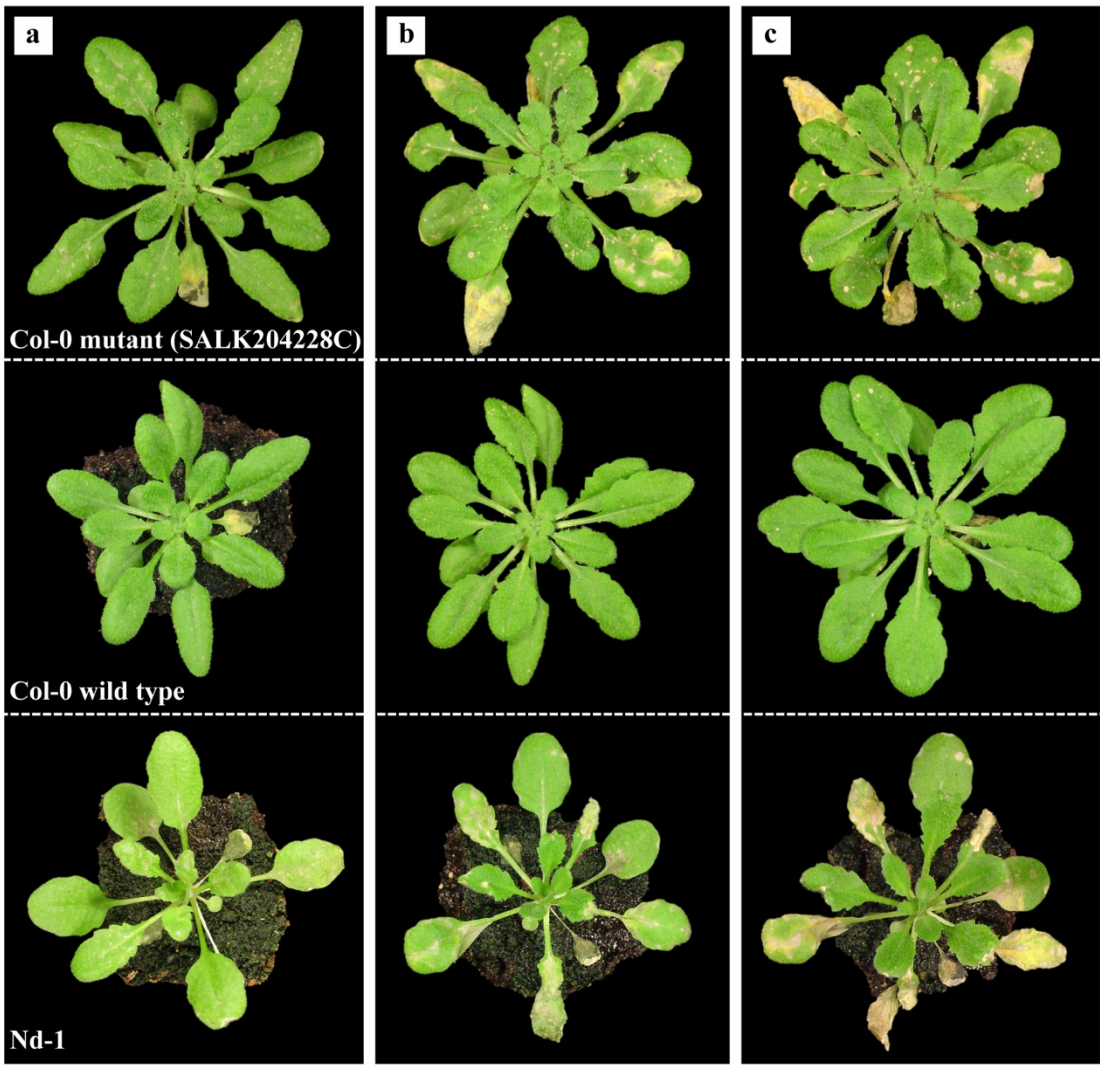



**Figure 14**. Interaction phenotypes of two standard accessions (Columbia, Col-0, and Niederzenz, Nd-1) of *Arabidopsis thaliana* and a Col-0 T-DNA insertion mutant (SALK204228C; insertion in coding region of At3g57710 gene) following spray inoculation with *Xanthomonas campestris* pv. *raphani* race 2 (strain HRI8305). Photographs were taken at different time-points after spray inoculation: **a**, 3 days after inoculation (dai); **b**, 5 dai; **c**, 7 dai.

To test the specificity of resistance conferred by the Columbia allele of At3g57710, the knockout mutant SALK204228C and the $F_9$ Col-0×Nd-1 recombinant inbred line 3790 (both susceptible to *Xcr* race 2 strain HRI8305; Table 17; Figure 14) were spray-inoculated with the type strains of *Xcr* races 1 and 3 (HRI6490 and HRI6519, respectively). Development of typical leaf spot symptoms were observed in plants of both lines following inoculations with HRI6519 (IP 3-4), whereas no symptom development was observed in plants inoculated with HRI6490 (IP 0-1) (four plants per line were tested with each strain in two experiment repeats).

These results suggest that *RXCR1* confers resistance at least to strains of two *Xcr* races (race 2 and 3).

Gain-of-function tests were performed initially by genetic transformation of the susceptible accession Nd-1 with three large overlapping JAtY clones (57K17, 64J11 and 62J13) of the resistant accession Col-0, which together span a 191.5 kb region in the predicted *RXCR1* interval delimited by markers m57350 and m58310 (Figure 13). The efficiency of *A. thaliana* transformation with JAtY clones was very low and only a one or two $T_1$ plants were obtained for each of the three clones after several attempts to obtain transgenic plants. The interaction phenotypes of the $T_3$ plants following inoculation with *Xcr* strain HRI8305 are given in Table 18. Gain-of-resistance (IP 0-2) was observed in all $T_3$ plants carrying the JAtY64J11 clone ($T_3$ Nd-1::JAtY64J11) (Figure 15, Table 18). This clone contains the candidate gene At3g57710 that was identified by fine-mapping and loss-of-function mutation (as described above). In contrast, Nd-1 lines transformed with either JAtY57K17 or JAtY62J11 ($T_3$ Nd-1::JAtY57K17 and $T_3$ Nd-1::JAtY62J11) did not show altered phenotype in comparison to susceptible control plants of Nd-1 wild type (Figure 15, Table 18).

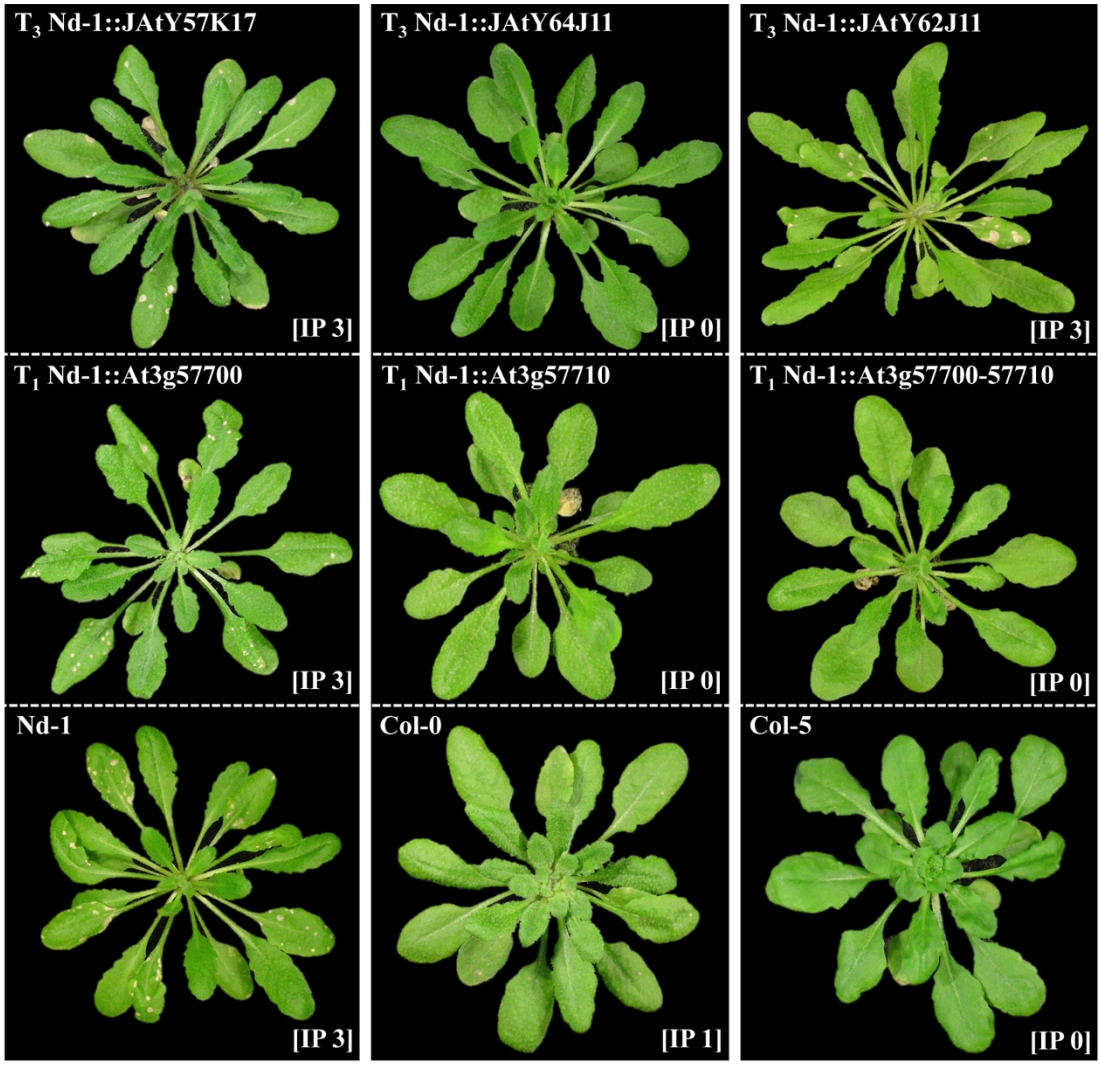



**Figure 15**. Interaction phenotypes of $T_3$ and $T_1$ transgenic *Arabidopsis thaliana* plants used to confirm gain-of-resistance to *Xanthomonas campestris* pv. *raphani* (race 2, strain HRI8305) conferred by the *RXCR1* allele (gene At3g57710) from Columbia. Niederzenz (Nd-1) was used as a susceptible background for genetic transformation with three JAtY clones (57K17, 64J11, 62J11) of large genomic fragments of Col-0 that span a 191.5 kb region in the map interval of *RXCR1* (as shown in Figure 13), and three clones from genomic fragments of Col-0 that contain genes At3g57700 and At3g57710 individually or in combination. Photographs were taken 7-10 days after spray inoculation with the pathogen. The interaction phenotype (IP) scores are indicated within brackets and were assessed based on a six-point phenotype scale (see Chapter 2, Figure 4).

**Table 18**. Summary of interaction phenotypes of transgenic plants of *Arabidopsis thaliana* following spray inoculation with *Xanthomonas campestris* pv. *raphani* strain HRI8305

| Transgenic plants[a] | Number of plants[b] | | |
|---|---|---|---|
| | Total | R[0-2] | S[3-5] |
| **JAtY clones** | | | |
| $T_3$ Nd-1::JAtY57K17 | 14 | | 14 |
| $T_3$ Nd-1::JAtY64J11 | 30 | 30 | |
| $T_3$ Nd-1::JAtY62J11 | 25 | | 25 |
| **Gene At3g57710** | | | |
| $T_1$ Nd-1::At3g57710 | 25 | 23 | 2 |
| $T_1$ SALK204228C::At3g57710 | 35 | 33 | 2 |
| $T_1$ 3790::At3g57710 | 19 | 18 | 1 |
| **Gene At3g57700** | | | |
| $T_1$ Nd-1::At3g57700 | 38 | | 38 |
| $T_1$ SALK204228C::At3g57700 | 14 | 3 | 11 |
| $T_1$ 3790::At3g57700 | 4 | | 4 |
| **Genes At3g57710 and At3g57700** | | | |
| $T_1$ Nd-1::At3g57700-At3g57710 | 58 | 56 | 2 |
| $T_1$ SALK204228C::At3g57700-At3g57710 | 55 | 55 | |
| $T_1$ 3790::At3g57700-At3g57710 | 28 | 28 | |
| **Controls** | | | |
| Col-0 | 6 | 6 | |
| Col-5 | 10 | 10 | |
| Nd-1 | 21 | 1 | 20 |
| SALK204228C (Col-0 mutant) | 6 | | 6 |
| 3790 ($F_9$ Col-0×Nd-1) | 6 | | 6 |

Abbreviations: R, resistant; S, susceptible.

[a] Transgenic plants were named with the plant accession background from which they derived followed by the respective accession of the JAtY clone or gene(s) contained in the clones used for transformation (described in Section 3.2.12). The respective filial generation of each plant ($T_1$ or $T_3$) precedes their name.

[b] Interaction phenotype scores are indicated within brackets and correspond to the maximum and minimum scores observed; the phenotypes were scored using a six-point scale presented in Chapter 2 (Figure 4).

For the second gain-of-function experiment, smaller clones containing either the gene At3g57710 or the neighbouring gene At3g57700 or both genes from Col-0, were transferred into three susceptible backgrounds, namely: Nd-1, the Col-0 knockout mutant SALK204228C and the $F_9$ Col-0×Nd-1 recombinant inbred line 3790. $T_1$ plants of each combination of accession and clone were tested for resistance to *Xcr* strain HRI8305 and the results are shown in Table 18. The majority

of the $T_1$ plants of all three backgrounds transformed with clones containing either At3g57710 alone or combined with At3g57700, were resistant (IP 0-2) (Table 18, Figure 15). In contrast, the majority of $T_1$ plants transformed with the clone containing At3g57700 alone were susceptible (IP 3-5) (Table 18, Figure 15). These results further confirm that the Col-0 allele of gene At3g57710 confers resistance to *Xcr* strain HRI8305.

### 3.3.5 *In planta* growth of *Xanthomonas campestris* pv. *raphani* strain HRI8305

The growth of *Xcr* race 2 strain HRI8305 in seven *A. thaliana* accessions was assessed by determining the average number of viable cells recovered from leaves over a period of seven days after spray inoculation (dai). The results obtained are presented in Figure 16. In the three accessions Nd-1, Col-0×Nd-1 recombinant inbred line 3790 and Col-0 knockout mutant SALK204228C, the bacterial density increased between 4 to 8-fold over 7 dai reaching a maximum of about $10^6$ cfu/cm$^2$. Over the same period, all plants of these accessions developed typical leaf spot symptoms (IP 3-5) as illustrated in Figure 14. In contrast, plants of the remaining four accessions tested showed complete absence of leaf spot symptoms (IP 0-1) or only a few small leaf spots (IP 2), over 7 dai as illustrated in Figure 14. The bacterial density decreased between 25 to 55-fold over the same period in the resistant accessions Col-0 and Col-5 (reaching a minimum near $10^3$ cfu/cm$^2$). A minor decrease of about 2.4 and 1.8-fold was observed in Oy-0 and Nd-1 transformed with the JAtY6411 clone (Nd-1::JAtYJ6411), respectively.

Significant differences were observed between the bacterial densities in the resistant Col-0 wild type and Col-0 knockout mutant SALK204228C, confirming that the gene disrupted in the Col-0 mutant (At3g57710) controls resistant to *Xcr* race 2 HRI8305. Over a period of 7 dai, the bacterial density increased about 9-fold in the Col-0 knockout mutant whereas in Col-0 wild type a decrease of nearly 55-fold was observed (Figure 16). Significant differences were also observed in the bacterial growth determined in the susceptible Nd-1 wild type and the transgenic line Nd-1::JAtYJ6411 that contains the At3g57710 allele from Col-0. In the transgenic Nd-1::JAtYJ6411, the bacterial density decreased slightly (about 1.8-fold over 7 dai)

whereas the bacterial density in the Nd-1 wild type increased about 8-fold (Figure 16).

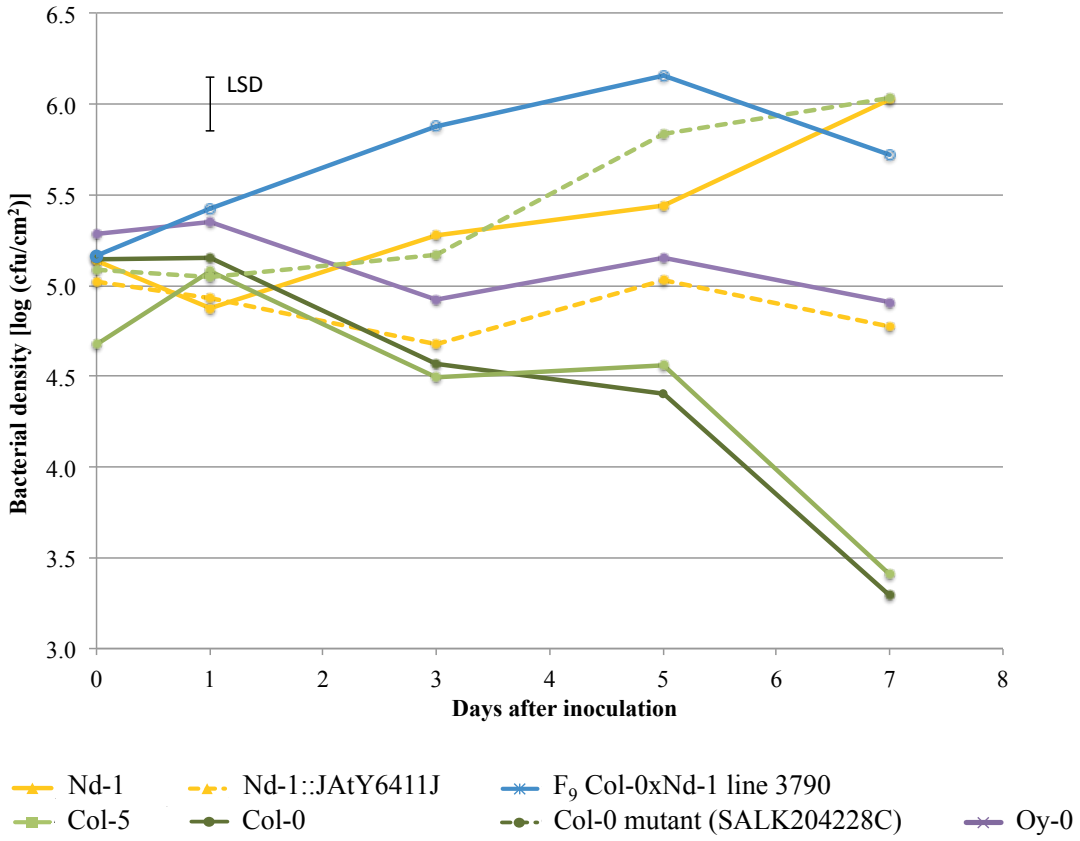



**Figure 16**. Bacterial growth profiles of *Xanthomonas campestris* pv. *raphani* race 2 strain HRI8305 in spray-inoculated *Arabidopsis thaliana* rosette leaves of three susceptible lines (Niederzenz, Nd-1; $F_9$ Col-0×Nd-1 recombinant inbred line 3790; and Col-0 knockout mutant SALK204228C) and three resistant lines (Columbia wild type, Col-0 and Col-5; Oystese, Oy-0, and a transgenic line Nd-1::JAtY6411C). Bacterial cells were recovered from leaves at the different time points indicated and colony-forming units (cfu) were counted by serial-dilution plating. The values at each time point represent the means of six plant replicates and four bacterial counts per plant replicate. LSD indicates the least significant difference between any two means (LSD 0.30; p-value 0.05). The standard error of the mean was 0.108.

The resistant controls, Col-0 and Col-5 (Col-0 *glabrous-1* mutant), showed no significant differences in the bacterial growth across the time period studied as expected, except immediately after inoculation (Figure 16). At this time-point, the bacterial density was significantly different between Col-0 and Col-5, which might be related to the presence of trichomes on the surface of leaves of Col-0 that potentially allowed retention of a greater number of bacterial cells than in the glabrous leaves of Col-5. It is interesting to highlight that *Xcr* race 2 HRI8305

showed two distinct growth profiles over a 7 dai period in the resistant controls Col-0/Col-5 and Oy-0 (which showed absence of typical leaf spot symptoms). A great reduction of bacterial density was observed in Col-0/Col-5 (about 25 to 55-fold) whereas only a minor reduction was observed in Oy-0 (about 2.4-fold).

### 3.3.6 *RXCR1* alleles of resistant and susceptible *Arabidopsis thaliana* accessions

The nucleotide sequences of *RXCR1* gene (At3g57710) annotated in the genome sequences of Col-0 and Nd-1 available at the *Arabidopsis* 1001 genomes database (http://signal.salk.edu/atg1001), were compared[1]. Ten SNPs were identified in the *RXCR1* sequence of Nd-1 and three cause amino acid changes (Table 19).

The *RXCR1* sequences of other *A. thaliana* accessions identified in Chapter 2 as resistant or susceptible to *Xcr* race 2 (strain HRI8305) and which had the complete genome sequence available at the *Arabidopsis* 1001 genomes database at the time of this research, were also compared (Table 19). A total of 19 SNPs were identified in susceptible accessions in comparison to Col-0 and formed 10 allelic variants that contain at least one SNP causing an amino acid change. The allelic variant of the resistant accession Col-0 was only found in some resistant accessions (*e.g.* the MAGIC parents Kn-0, No-0, Tsu-0 and Wil-2). Among the remaining resistant accessions examined, some shared allelic variants with susceptible accessions as for example the MAGIC parents Oy-0, Edi-0 and Ws-0 that were predicted to have an allele for resistance in the *RXCR2* locus on chromosome 5 (see Section 3.3.1, Figure 10b).

Sequence variation was also observed among resistance and susceptible accessions in the intragenic region between *RXCR1* (At3g57710) and At3g57720 that contains the promoter region of *RXCR1*. In comparison to Col-0, sequence variation was mainly observed in the accessions that also contained SNPs in the *RXCR1* sequence (Appendix 9).

[1] The complete genome of the *A. thaliana* accession Nd-1 became publically available towards the end of this study, after fine-mapping of *RXCR1* had been completed.

**Table 19**. Summary of sequence polymorphisms in the *RXCR1* gene (At3g57710) of a collection of *Arabidopsis thaliana* accessions that are resistant (green highlighting) or susceptible (yellow highlighting) to *Xanthomonas campestris* pv. *raphani* strain HRI8305 [a]

| Position in chromosome 3 | | 21,386,224 | 21,386,207 | 21,386,195 | 21,386,177 | 21,386,162 | 21,386,151 | 21,386,138 | 21,386,129 | 21,386,066 | 21,386,048 | 21,386,018 | 21,386,010 | 21,385,783 | 21,385,665 | 21,385,634 | 21,385,631 | 21,385,602 | 21,385,425 | 21,385,314 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Position in coding sequence (5'-3')** | | 10 | 27 | 39 | 57 | 72 | 83 | 96 | 105 | 168 | 186 | 216 | 224 | 451 | 569 | 600 | 603 | 632 | 809 | 920 |
| **Accession** | **Allelic variants** | **Nucleotide[a]** | | | | | | | | | | | | | | | | | | |
| Col-0 | 1 | C | A | G | G | G | G | A | A | C | T | T | A | G | G | C | G | C | C | G |
| Nd-1 | 2 | . | G | A | A | A | A | T | T | T | A | . | . | . | A | . | . | . | . | . |
| Del-10 | 3 | . | G | A | A | A | A | T | T | T | A | G | . | . | . | . | . | . | . | . |
| ICE73 | | . | G | A | A | A | A | T | T | T | A | G | . | . | . | . | . | . | . | . |
| Bak-7 | 4 | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | . |
| Ct-1 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | . |
| ICE7 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | . |
| ICE97 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | . |
| ICE104 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | . |
| ICE107 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | . |
| ICE112 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | . |
| ICE169 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | . |
| ICE173 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | . |
| ICE181 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | . |
| Sha | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | . |
| Ey-15-2 | 5 | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | A |
| ICE226 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | A |
| ICE228 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | A |
| Rue3-1-31 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | A |
| Vash-1 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | A |
| Po-0 | 5/6 | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | T/C | . | A/G |
| Lerik1-3 | 6 | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | T | . | . |
| ICE98 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | T | . | . |
| ICE106 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | T | . | . |
| Sf-2 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | T | . | . |
| Star-8 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | T | . | . |
| Zu-0 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | T | . | . |
| ICE92 | 7 | . | G | A | A | A | A | T | T | T | A | . | . | . | . | A | A | T | . | . |
| ICE119 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | A | A | T | . | . |
| Cvi-0 | 8 | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | T | . | . |
| Dog-4 | 9 | T | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . |
| ICE79 | | T | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . |
| Kastel-1 | | T | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . |
| TueSB30-3 | | T | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . |
| Tuescha9 | | T | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . |
| ICE50 | 10 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . |
| ICE61 | | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . |
| Qui-0 | 11 | . | . | . | . | . | . | . | . | . | . | . | . | A | . | . | . | . | T | . |
| Oy-0 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | A |
| Edi-0 | 5 | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | A |
| Hh-0 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | A |
| TueV13 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | A |
| ICE72 | 4 | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | . |
| ICE75 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | . |
| Mer-6 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | . |
| Ws-0 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | . |
| Yeg-1 | | . | G | A | A | A | A | T | T | T | A | . | . | . | . | . | . | . | . | . |
| Haes-1 | 9 | T | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . |
| WalhaesB4 | | T | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . |
| ICE127 | 10 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . |
| ICE130 | | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . |
| ICE150 | | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . |
| ICE152 | | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | . | . |
| Bak-2 | 1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| C24 | | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| ICE1 | | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| ICE33 | | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| ICE63 | | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| ICE120 | | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| ICE212 | | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| ICE213 | | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Kn-0 | | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Koch-1 | | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| No-0 | | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Tsu-0 | | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Tsu-1 | | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Wil-2 | | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |

[a] Sequence polymorphisms are indicated in comparison to the resistance *RXCR1* allele of Columbia (Col-0). Mutations that introduce amino acid changes are highlighted in red and mutations that introduce stop codons are highlighted in black. All nucleotide sequences compared were retrieved from the *Arabidopsis* 1001 genomes database (http://signal.salk.edu/atg1001/3.0/gebrowser.php).

## 3.4 DISCUSSION

Phenotypic characterization of interactions between a diverse collection of *A. thaliana* accessions and bacterial strains representing three known races of *Xcr* revealed examples of broad susceptibility to all three races, genotype specific resistance (to some, but not all races), and broad resistance to all three races (Chapter 2). Nineteen of the accessions used in this phenotyping study have been used as parents to generate the genetic mapping resource of multi-parented MAGIC inbreds (Kover *et al.*, 2009), which proved to be an excellent tool to investigate genetic loci for resistance to *Xcr*. This resource enabled the identification of two loci controlling resistance to *Xcr* race 2 strain HRI8305: one on the bottom arm of chromosome 3 (designated *RXCR1*; map interval 20,26 - 23,29 Mb) and another on the bottom arm of chromosome 5 (designated *RXCR2*; map interval 16,43 - 22,7 Mb). Further fine-mapping within the *RXCR1* locus and molecular verification experiments (described below) led to the identification of a single gene controlling resistance in Columbia (At3g57710) to *Xcr*.

The identification of *RXCR1* (gene At3g57710) involved several steps. The map interval determined using the MAGIC population, was refined to 1.87 Mb by analysing additional genotype and phenotype data that was available for a two-parent mapping population ($F_9$ Col×Nd-1). New markers were then used to identify $F_9$ and $F_2$ lines which showed recombination within this map interval thus allowing to define a narrower interval of 62.31 kb containing 20 candidate genes. For molecular verification, Col-0 knockout mutants of several candidate genes were tested for response to *Xcr* race 2, and only the mutant of gene At3g57710 showed loss-of-resistance (full symptom development and an increase in bacterial density similar to the one observed in the susceptible accession Nd-1, Figure 16). For gain-of-function assays, three clones of genomic DNA from the resistant Col-0 accession (JAtY clones) that span the target interval were transferred into the susceptible accession Nd-1. Only the clone that contained At3g57710 showed gain-of-function (absence of symptom development and a significant decrease in bacterial density in comparison to the wild-type, Figure 16), further confirming the results from the loss-of-function experiment. Gain-of-resistance was also observed in the majority of $T_1$ plants derived from three additional susceptible backgrounds, including Nd-1, Col-0

knockout mutant of At3g57710 and the Col-0×Nd-1 recombinant inbred line 3790 (predicted to have the Nd-1 allele of *RXCR1*, Table 17), which were transformed with clones containing either the Col-0 allele of At3g57710 alone or combined with the Col-0 allele of At3g57700. The majority of $T_1$ plants transformed with the Col-0 allele of At3g57700 alone were susceptible confirming that this gene does not control resistance. Only $T_1$ plants were tested for resistance to HRI8305 due to time constraints, however, the combined results from these multiple and independent transformation experiments provide conclusive evidence for gain-of-resistance from a single transgene.

The *RXCR1* gene alone is insufficient to explain resistance in Columbia to all three races of *Xcr*. The Col-0 knockout mutant of gene At3g57710 and the Col-0×Nd-1 RIL 3790 (predicted to have the *RXCR1* susceptible allele from Nd-1; Table 17) were both susceptible to the reference strains of *Xcr* races 2 (HRI8305) and 3 (HRI6519), but not to race 1 (HRI6490). These findings suggest that *RXCR1* also controls resistance to *Xcr* race 3 but other gene(s) are required for resistance to *Xcr* race 1. Phenotyping the $F_9$ Col×Nd-1 mapping population for response to HRI6490 will be useful to map loci conferring resistance to *Xcr* race 1 in Columbia.

The *RXCR1* gene (At3g57710) was also identified to confer resistance in *A. thaliana* to *X. campestris* in an independent study (Huard-Chauveau *et al.*, 2013), which was published after completion of the experimental work presented in this Chapter. The authors named this gene *RKS1* (Resistance related kinase 1) and showed that the Columbia allele confers resistance to several races of *X. campestris* pv. *campestris* (races 1, 3, 5, 7 and 9). This gene was also described to confer resistance to other pathovars of *X. campestris* including *raphani*, *armoraciae* and *incanae*. In that study, the *RKS1* mediated resistance to *Xcr* was confirmed using two strains of *Xcr* that have been identified as race 3 (756C and CFBP5828; Fargier & Manceau, 2007), and these results together with the results of the present study confirm that this gene mediates resistance to strains of at least two *Xcr* races (races 2 and 3).

Progress has been made to begin elucidating the role of *RXCR1/RKS1* in plant defence. This gene encodes a kinase-like protein and it is located within a cluster of eight genes encoding highly similar proteins. ZED1 (At3g57750) encoded by the sixth gene in this cluster was reported to be required for resistance to *P. syringae*

(Lewis *et al.*, 2013). Wang *et al.* (2015) reported that RKS1/RXCR1 interacts with the nucleotide-binding leucine rich repeat (NB-LRR) receptor protein ZAR1 (HopZ-activated resistance 1), for detection of the *Xcc* pathogen effector AvrAC. AvrAC targets plant receptor-like cytoplasmic kinases (RLCKs) to suppress plant immunity and promote virulence (Feng *et al.*, 2012). The RLCK PBL2 is also modified by AvrAC, and RKS1 has been shown to form a complex with ZAR1 that recruits the modified PBL2 for immune response (Wang *et al.*, 2015). Interestingly, ZED1 was described to play a similar role in pathogen detection and interacts with ZAR1 for recognition of the *P. syringae* effector HopZ1a (Lewis *et al.*, 2013).

Sequence analysis of the *RXCR1* gene from resistant and susceptible *A. thaliana* accessions to *Xcr* race 2 (strain HRI8305), revealed variation in comparison to the functional resistance allele in Col-0. The accession Nd-1 that was used as the susceptible parent for interval mapping of *RXCR1*, has a block of variation in the coding sequence that is conserved among the majority of the susceptible accessions compared (alleles 2-7, Table 19). This block correlates with another block of sequence variation in the 5' upstream region of the gene relative to the Columbia allele (data shown in Appendix 9). It is not known whether this variation affects gene expression and/or function of the encoded protein. However, it is interesting to note that these blocks of sequence variation are also present in some resistant accessions (*e.g.* the MAGIC parent Oy-0) and if this variation were correlated with the *RXCR1* loss-of-function, those accessions would have a different locus conferring resistance. This observation is in line with the results for resistance in Oy-0 discussed below.

As stated above, interval mapping using the MAGIC population also indicated a second locus designated *RXCR2* on the bottom arm of chromosome 5, and Oy-0 was predicted as a representative accession that has an allele at this locus for resistance to *Xcr* race 2 (strain HRI8305). Using a bi-parental $F_8$ mapping population derived from a cross between Col-0 and Oy-0, two susceptible lines were identified thus indicating that resistance in these two parents is controlled by different loci. Furthermore, the bacterial growth profiles also differed in these accessions during the first 7 days after inoculation, further suggesting that a different resistance mechanism may be involved. In Col-0, the bacterial density was restricted to low levels whereas in Oy-0, higher bacterial levels were tolerated (Figure 16).

The map location of the *RXCR2* in Oy-0 was confirmed by Sonawala (2013) using a $F_9$ Nd-1×Oy-0 inbred population, to a similar region on the bottom arm of chromosome 5 (15.9 - 22.8 Mb) as predicted by the mapping analysis using the MAGIC population. Moreover, no additional resistance locus was mapped to the *RXCR1* locus further confirming that *RXCR1* does not provide resistance to *Xcr* race 2 in this accession. The *RXCR2* map interval identified in the present study includes a genomic region that contains several NB-LRR genes including molecularly characterized disease resistance genes (*e.g.* the gene pair *RPS4*/*RRS1*, *RPS6* and *RPP8*) (Holub, 2001; Meyers *et al.*, 2003). Resistance to *Xcc* has been mapped within this interval in an early study by Buell & Somerville (1997) (*RXC3* locus mapped approximately from 17.8 to 19.0 Mb based on physical positions of the nearest markers available in TAIR, http://www.arabidopsis.org). And recently, the gene pair *RPS4* (Resistance to *Pseudomonas syringae* 4) and *RRS1* (Resistance to *Ralstonia solanacearum* 1) which is located within the *RXCR2* map interval and has been described to confer resistance to strains of the bacterial pathogens *P. syringae* and *Ralstonia solanacearum* and a fungal pathogen (Gassmann *et al.*, 1999; Deslandes *et al.*, 2003; Narusaka *et al.*, 2009), was also reported to confer resistance to a strain of *Xcc* race 6 in Ws-0 (Debieu *et al.*, 2016). In that study, a Ws-0 double mutant of the *RPS4* and *RRS1* genes was also tested with strains of *Xcr*, but no evidence for the involvement of this gene pair in resistance to *Xcr* was found. The authors assessed the interactions between the Ws-0 double mutant of *RPS4* and *RRS1* with two *Xcr* strains that have been assigned to race 3 (756C and CFBP5828) (Fargier & Manceau, 2007) and a strain of unknown race (CFBP7144) which is listed as unauthenticated *Xcr* strain in the CFBP catalogue (http://catalogue-cfbp.inra.fr/resultnum_e.php?r0=7144). According to the results obtained in the present study (Table 7, Chapter 1), Ws-0 is susceptible to at least four strains of *Xcr* race 3 (including the strain 756C or HRI8503 also tested by Debieu *et al*. (2016)) and therefore the *RPS4/RRS1* alleles of Ws-0 may not confer resistance to strains of this race. However, the results of the present study indicated that Ws-0 is resistant to strains of *Xcr* races 1 and 2 and therefore the Ws-0 double mutant of *RPS4* and *RRS1* genes could be tested in future research to determine whether this gene pair is involved in resistance to strains of these races. Moreover, the fine-mapping of the *RXCR2* locus could be achieved in future research by developing more markers within the interval using the $F_9$ Nd-1×Oy-0 inbred population. This would provide a basis for cloning gene(s) controlling resistance to *Xcr* in Oy-0.

# CHAPTER 4. Characterization of *Xanthomonas campestris* strains associated with outbreaks of a leaf spot and blight disease in vegetable brassicas in Mauritius

## 4.1 INTRODUCTION

Severe outbreaks of an atypical leaf spot and blight disease of brassica crops have occurred in Mauritius and have been associated with *Xanthomonas campestris* (R. Lobin, pers. comm.). The disease has been observed since 2009 in vegetable production regions characterized by high temperature and humidity, leading to crop losses of up to 100%. In South Carolina (USA), similar outbreaks occurred in vegetable brassica crops grown in Lexington County between 2001 and 2007, causing total economic losses estimated at $1.7 million (Wechter *et al.*, 2008). In both regions, the symptoms began as small brown necrotic spots that rapidly expanded and coalesced to cover large areas of the leaves. These symptoms differed from the typical black rot symptoms caused by *X. campestris* pv. *campestris* (*Xcc*) that are characterized by V-shaped lesions often starting at the margin of the leaves and that are commonly observed in brassica crops grown in those regions (Wechter *et al.*, 2008; R. Lobin, pers. comm.).

Atypical disease symptoms caused by *X. campestris* strains on *Brassica* spp. have also been described in earlier reports. For example, Knösel (1961a) described atypical leaf spot symptoms in cauliflower and proposed a new pathogenic variant, *aberrans*, but Vicente *et al.* (2001) included the type strain of this pathovar into *Xcc* race 5 based on disease symptoms and interactions with *Brassica* differential lines. Leaf spot symptoms that progressed into large necrotic areas were reported by Moffett *et al.* (1976) in cauliflower, cabbage and broccoli. Yuen & Alvarez (1985) observed a distinctive rapid necrosis of the leaf tissues in cabbage (described as leaf blight) and Alvarez *et al.* (1994) suggested that blight causing *X. campestris* strains might constitute an aggressive variant of *Xcc.* More recently, Gaetan & Lopez (2005) reported an outbreak in Argentine of a leaf spot disease of canola (*B. napus*), associated to *X. campestris* and characterized by severe necrosis of leaves and defoliation in advanced phases of the disease.

Besides *X. campestris*, pseudomonads are known to cause leaf spot and blight diseases on *Brassicas* spp. and include: *Pseudomonas cichorii* (Wehlburg, 1963), *P. syringae* pv. *maculicola* (Zhao *et al.*, 2000b; Keinath *et al.*, 2006; Takikawa & Takahashi, 2014) and *P. syringae* pv. *alisalensis* that has been amended to *P.*

*cannabina* pv. *alisalensis* (Cintas *et al.*, 2002; Bull *et al.*, 2010b; Takikawa & Takahashi, 2014).

Strains from South Carolina were identified as *Xcc* and were distinguished from the leaf spotting pathogen *X. campestris* pv. *raphani* (*Xcr*) in pathogenicity assays (Wechter *et al.*, 2008). In Mauritius, further investigation was needed to determine whether the causal pathogen(s) of the atypical symptoms were *Xcc* and/or other pathovar such as *Xcr* and whether *Pseudomonas* sp. could also be involved in the disease. Thus, the aim of the present Chapter was to identify and characterize bacterial strains isolated from brassicas showing leaf spot and blight symptoms in Mauritius and to compare them with strains isolated from outbreaks in South Carolina.

Specific objectives were to:

a) Identify and characterize strains isolated from leaf samples collected in Mauritius in 2009 and 2012, using partial sequences of two conserved genes, *16S* (encoding the *16S* ribosomal RNA) and *gyrB* (encoding the subunit B protein of DNA gyrase), and compare them with *Xcc* strains from South Carolina. The *16S* gene was chosen because it is commonly used for identification of bacterial strains at least to the genus level (Janda & Abbott, 2007); the *gyrB* gene was chosen because it has been described as useful for identification of *Xanthomonas* strains to the species level and discrimination of strains within species (Parkinson *et al.*, 2009).

b) Determine whether strains from both regions were similar in their pattern of host reactions (under glasshouse conditions) to any of the previously designated races of *Xcc* and/or *Xcr*, using a standard host differential (including several *Brassica* spp. and radish) developed for distinguishing races of *Xcc* and *Xcr* (Vicente *et al.*, 2006; Vicente & Holub, 2013).

Additionally, the present Chapter includes characterization of pathogenicity and/or race determination of the following strains:

a) The pathotype strain of *Xcr* (NCPPB1946) that was possibly wrongly linked to a *Xcc* strain present in the collection of the University of Warwick Crop Centre (Vicente *et al*., 2001; 2006).

b) Five strains received as *Xcr* of unknown race from: the UK (D. Stead and J. Vicente, pers. comm.), Italy (Loreti *et al.*, 2011), Africa (J. Mulema, pers. comm.) and India (National Collection of Plant Pathogenic Bacteria, Fera, York, UK).

## 4.2 MATERIALS AND METHODS

### 4.2.1 Isolation of bacteria from leaf samples

Leaf samples from cauliflower plants grown in the Central Plateau region of Mauritius in October of 2012 showing leaf spot and blight symptoms, were collected by R. Lobin (Agricultural Research and Extension Unit, Plant Pathology Division, Mauritius). Small leaf sections were cut with a sterile scalpel, washed and ground with sterile distilled water. The tissue suspensions were streaked on King's B agar plates (King *et al.*, 1954) and yeast dextrose calcium carbonate (YDC) agar plates (Schaad *et al.*, 2001) (recipes provided in Appendix 2). Plates were incubated for 48 h at 28 °C.

Bacterial colonies grown on agar media were examined. On King's B medium, colonies were observed under UV-light (365 nm) to detect the presence of fluorescent bacteria. In order to obtain pure bacterial cultures, one isolated colony of each type was re-streaked on King's B medium and incubated for 48 h at 28 °C. Bacterial cultures were then preserved at −76 °C in Nutrient Broth (Difco) (8 g/l) with 15% (w/v) glycerol as described by Feltham *et al.* (1978). All bacterial cultures obtained were added to the bacterial collection of the University of Warwick Crop Centre (HRI collection, Wellesbourne, UK).

### 4.2.2 Bacterial strains

All strains studied in this Chapter are listed in Table 20. They include strains isolated from *Brassica* spp. with leaf spot and blight symptoms grown in Mauritius and South Carolina (USA), namely: six *Xanthomonas* and five *Pseudomonas* strains that were isolated in this study from leaf samples collected in Mauritius in 2012, four *Xanthomonas* strains isolated from leaf samples collected in Mauritius in 2009 (provided by R. Lobin and J. Vicente), and three strains from South Carolina previously identified as *Xcc* (Wechter *et al.*, 2008) and provided by P. Wechter (USDA-ARS, Charleston, South Carolina, USA).

Six additional strains were used for pathogenicity tests and included: the pathotype strain of *Xcr* obtained from the National Collection of Plant Pathogenic

Bacteria (NCPPB, Fera, York, UK; strain NCPPB1946, HRI8803), and five strains received as *Xcr* of unknown race from the UK (strain HRI8474 provided by D. Stead and J. Vicente), Italy (strains HRI8834 and 8835 provided by S. Loreti, CRA-PAV, Rome, Italy), Africa (strain HRI7803 obtained from the HRI collection) and India (strain HRI8804 obtained from the NCPPB).

**Table 20**. List of bacterial strains studied

| HRI strain accession (other designation) | Isolation | | | Source (reference[a]) |
|---|---|---|---|---|
| | Host | Geographical origin | Year | |
| ***Xanthomonas* strains isolated from field-grown *Brassica* spp. with leaf spot and blight symptoms** | | | | |
| 8506 (A) | *B. oleracea* var. *capitata* (cabbage) | Mauritius | 2009 | R. Lobin |
| 8507 (B) | *B. oleracea* var. *capitata* (cabbage) | Mauritius | 2009 | R. Lobin |
| 8514 | *B. oleracea* var. *botrytis* (cauliflower) | Mauritius | 2009 | J. Vicente and R. Lobin |
| 8516A | *B. oleracea* var. *capitata* (cabbage) | Mauritius | 2009 | J. Vicente and R. Lobin |
| 8806 (418) | *B. juncea* cv. Green Wave (mustard) | Pelion, South Carolina, USA | 2005 | P. Wechter (3) |
| 8807 (543) | *B. rapa* cv. Topper (turnip) | Lexington, South Carolina, USA | 2006 | P. Wechter (3) |
| 8808 (711) | *B. juncea* Plant Introduction | Charleston, South Carolina, USA | 2007 | P. Wechter (3) |
| 8821 (2A) | *B. oleracea* var. *botrytis* (cauliflower[b]) | Mauritius | 2012 | This study |
| 8823 (6A) | *B. oleracea* var. *botrytis* (cauliflower[b]) | Mauritius | 2012 | This study |
| 8824A (7A) | *B. oleracea* var. *botrytis* (cauliflower[b]) | Mauritius | 2012 | This study |
| 8825 (8A) | *B. oleracea* var. *botrytis* (cauliflower[b]) | Mauritius | 2012 | This study |
| 8826A (9A) | *B. oleracea* var. *botrytis* (cauliflower[b]) | Mauritius | 2012 | This study |
| 8827A (11A) | *B. oleracea* var. *botrytis* (cauliflower[b]) | Mauritius | 2012 | This study |
| ***Pseudomonas* strains isolated from field-grown cauliflowers with leaf spot and blight symptoms** | | | | |
| 8822A (4A) | *B. oleracea* var. *botrytis* (cauliflower[b]) | Mauritius | 2012 | This study |
| 8822B (4B) | *B. oleracea* var. *botrytis* (cauliflower[b]) | Mauritius | 2012 | This study |
| 8824B (7B) | *B. oleracea* var. *botrytis* (cauliflower[b]) | Mauritius | 2012 | This study |
| 8827B (11B1) | *B. oleracea* var. *botrytis* (cauliflower[b]) | Mauritius | 2012 | This study |
| 8827C (11B2) | *B. oleracea* var. *botrytis* (cauliflower[b]) | Mauritius | 2012 | This study |
| **Other strains** | | | | |
| *Xanthomonas campestris* pv. *raphani* | | | | |
| 7803 (BD63) | *B. oleracea* var. *capitata* (cabbage) | Brits, South Africa | 1997 | HRI (2) |
| 8474 (P764, NCPPB4451) | *Erysimum cheiri* (wallflower) | UK | na | NCPPB, D. Stead and J. Vicente |
| 8803[Pt] (NCPPB1946) | *Raphanus sativus* (radish) | USA | 1940 | NCPPB, 2010 |
| 8834 (1524) | *R. sativus* (radish) | Latium region, Italy | 2009 | S. Loreti (1) |
| 8835 (1526) | *R. sativus* (radish) | Latium region, Italy | 2009 | S. Loreti (1) |
| *X. campestris* pv. *campestris* (received as *X. campestris* pv. *raphani*) | | | | |
| 8804 (NCPPB3566) | *R. sativus* (radish) | India | na | NCPPB |

Abbreviations: HRI, formerly Horticulture Research International, now University of Warwick Crop Centre, Wellesbourne, UK; na, not available; NCPPB, National Collection of Plant Pathogenic Bacteria, Fera, York, UK; Pt, pathotype.

[a] 1, Loreti *et al.* (2011); 2, Vicente *et al.* (2001); 3, Wechter *et al.* (2008).

[b] Cauliflower cv. Local: an open-pollinated cultivar collected in the central plateau region of Mauritius (R. Lobin, pers. comm.).

For comparative purpose, eight reference strains listed in Table 21 were also used. They included the type strains of *Xcc* races 4, 5 and 6, *Xcr* races 1, 2 and 3, as well as the *Xcc* race 5 strain HRI3883 that has been deposited in the HRI collection as a clone of the pathotype strain of *Xcr* (NCPPB1946), but it has been identified as *Xcc* race 5 (Vicente *et al.*, 2001, 2006).

**Table 21**. List of reference strains of *Xanthomonas campestris* used in this study

| HRI strain accession (other designation) | Isolation | | | Reference[a] |
|---|---|---|---|---|
| | Host | Geographical origin | Year | |
| ***X. campestris* pv. *campestris*** | | | | |
| **RACE 4** | | | | |
| 1279A$^{Rt}$ | *B. oleracea* var. *capitata* (cabbage) | Cornwall, UK | 1984 | 1 |
| **RACE 5** | | | | |
| 3880$^{Rt, b}$ | *B. oleracea* var. *capitata* (cabbage) | Australia | 1975 | 1, 2 |
| 3883 | na | na | na | 1, 2 |
| **RACE 6** | | | | |
| 6181$^{Rt}$ (Xcc551) | *B. rapa* | Sardoal, Portugal | 1996 | 1 |
| ***X. campestris* pv. *raphani*** | | | | |
| **RACE 1** | | | | |
| 6490$^{Rt}$ (P5034) | *B. oleracea* var. *botrytis* (cauliflower) | France | 1995 | 2 |
| **RACE 2** | | | | |
| 8305$^{Rt}$ (BR-25) | *B. rapa* var. *perviridis* (spinach mustard) | Oklahoma, USA | 1995 | 2 |
| **RACE 3** | | | | |
| 6519$^{Rt}$ (TMR74) | *Raphanus sativus* (radish) | Shizuoka, Japan | 1985 | 2 |

Abbreviations: HRI, formerly Horticulture Research International, now University of Warwick Crop Centre, Wellesbourne, UK; na, not available; NCPPB, National Collection of Plant Pathogenic Bacteria, Fera, York, UK; Pt, pathotype strain; Rt, race-type strain.

[a] References: 1, Vicente *et al.* (2001); 2, Vicente *et al.* (2006).

[b] Pathotype strain of *X. campestris* pv. *aberrans* (HRI3880) which has been defined as the type strain of *X. campestris* pv. *campestris* race 5 by Vicente *et al.* (2001).

### 4.2.3 DNA amplification and sequencing

Bacterial suspensions were prepared in sterile ultrapure water and the concentration adjusted visually to the McFarland turbidity standards 3 to 4 (*ca*. $10^8$ cfu/ml) (Király *et al.*, 1974). Templates for Polymerase Chain Reaction (PCR) were prepared from bacterial suspensions by freezing and thawing to promoted cell lysis and DNA release.

All PCR amplifications of fragments of the *gyrB* and *16S* genes, were carried out by using a blend of *Taq* DNA Polymerase and a DNA polymerase with proofreading activity (Long PCR Enzyme mix; Fermentas), to avoid errors specially in amplification of DNA of xanthomonads that have a very high GC content

(Parkinson *et al.*, 2007). The reaction mixtures comprised a total volume of 25 µl and contained 1× reaction buffer (without $MgCl_2$), 0.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 µM of each primer of each primer, 0.025 U/µl Long PCR Enzyme mix (Fermentas), and 2.5 µl of DNA template. The primers used are listed in Table 22. Primers XgyrB1F and XgyrB1R, were used to amplify a fragment of the *gyrB* gene (Young *et al.*, 2008) and primers 27f and 1492r were used to amplify a fragment of the *16S* gene (Lane, 1991).

**Table 22**. Primers used to amplify fragments of *16S* and *gyrB* genes

| Gene | Primer | Sequence (5' to 3') | Expected amplified fragment size (bp) | Reference |
|---|---|---|---|---|
| *gyrB* | XgyrB1F | ACGAGTACAACCCGGACAA | 900 | Young *et al.* (2008) |
| | XgyrB1R | CCCATCARGGTGCTGAAGAT | | |
| *16S* | 27f | AGAGTTTGATCMTGGCTCAG | 1500 | Lane (1991) |
| | 1492r | GGTTACCTTGTTACGACTT | | |

The PCR program used was the same as the one published by Young *et al.* (2008): an initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The purity and yield of PCR products were checked by electrophoresis of 5 µl of reaction mixture on a 1% (w/v) agarose gel with 0.006% (v/v) GelRed nucleic acid gel stain (Biotium), at 100 V for 1 h. A volume 6.25 µl of 1 Kb Plus DNA ladder (50 ng/µl; Invitrogen) was run in one well of each gel to provide a reference for DNA fragment size and concentration (25 ng/µl at 1650 bp band). Gels were visualized under UV light.

PCR products were purified using the Qiaquick PCR Purification kit (Qiagen) and following the manufacturer's instructions. Purified products were sequenced by a commercial service (GATC Biotech) in both directions using the same forward and reverse primers used for PCR amplifications.

Nucleotide sequences were checked, edited and aligned using the SeqMan program of the DNAstar Lasergene 9 software package (DNAstar). Low-quality ends of each sequence were trimmed. Forward and reverse sequences of each PCR product were aligned to generate a consensus sequence.

### 4.2.4 Sequence similarity searches

Similarity searches of *16S* gene sequences obtained in this study were performed against the public nucleotide database of NCBI (http://www.ncbi.nlm.nih.gov) using the BLASTN program (Altschul *et al.*, 1990) implemented in Geneious software (Kearse *et al.*, 2012).

### 4.2.5 Phylogenetic analysis

Phylogenetic trees were constructed based on nucleotide sequences of individual genes (*16S* or *gyrB*), obtained in this study and retrieved from the nucleotide database of NCBI (http://www.ncbi.nlm.nih.gov). Sequences of each gene were aligned and trimmed to a common start and end nucleotide position using Geneious software. Sequence variation was examined using the same software. Phylogenetic analyses were conducted using MEGA6 software (Tamura *et al.*, 2013). The model that best described the nucleotide substitution pattern of each gene sequence dataset was selected using the same software. Phylogenetic trees were constructed using the Maximum Likelihood method (Felsenstein, 1981) based on the selected substitution model. The reliability of each node in the trees was estimated with 1000 bootstrap replicates (Felsenstein, 1985).

### 4.2.6 Plant materials and growth conditions

Plants used in this study are listed in Table 23. They included the *Brassicaceae* host differentials of *Xcc* and *Xcr* races (Vicente *et al.*, 2006; Vicente & Holub, 2013; summarized in Table 2, Chapter 1), cauliflower cv. Local from Mauritius and tomato cv. Moneymaker (susceptible control for *Xcr* strains; Vicente *et al*., 2006).

Seeds were sown in squared 7×7×8 cm pots with Levington M2 compost (Everris) treated with 0.28 mg/litre of Intercept 5GR (active ingredient imidacloprid) (Everris). Two plants were grown in each pot and maintained in a glasshouse at 20 to 22 °C under a photoperiod of 10 h.

**Table 23**. Plants used for pathogenicity tests and race typing of *Xanthomonas campestris* pv. *raphani* and *Xanthomonas campestris* pv. *campestris* strains

| Species / variety | Cultivar / line | Type | Source |
|---|---|---|---|
| *Brassica oleracea* | line SxD1 | Doubled haploid obtained by microspore culture from a plant derived from a cross between *B. oleracea* cv. Böhmerwaldkohl and a rapid cycling line | University of Warwick Crop Centre, UK |
| **Cabbage** | | | |
| *B. oleracea* var. *sabauda* (Savoy cabbage) | cv. Wirosa | $F_1$ hybrid | Bejo, Netherlands |
| **Cauliflower** | | | |
| *B. oleracea* var. *botrytis* | cv. Local | Open-pollinated cultivar collected in the central plateau region of Mauritius | R. Lobin |
| *B. oleracea* var. *botrytis* | cv. Miracle | $F_1$ hybrid | Bejo, Netherlands |
| **Mustards** | | | |
| *B. carinata* | line PIC1 | Doubled haploid obtained by microspore culture from a selection of *B. carinata* accession PI199947 (Ethiopian mustard) | University of Warwick Crop Centre, UK |
| *B. juncea* | line FBLM2 | Doubled haploid obtained by microspore culture from a plant of *B. juncea* cv. Florida Broad Leaf Mustard | University of Warwick Crop Centre, UK |
| **Oilseed rape** | | | |
| *B. napus* var. *oleifera* | line COB60 | Doubled haploid obtained by microspore culture from a selection of *B. napus* cv. Cobra line 14R | University of Warwick Crop Centre, UK |
| **Turnip** | | | |
| *B. rapa* var. *rapifera* | cv. Just Right | Hybrid | Otis S. Twilley Seed, USA |
| *B. rapa* var. *rapifera* | cv. Seven Top | Open-pollinated | Otis S. Twilley Seed, USA |
| **Radish** | | | |
| *Raphanus sativus* | cv. French Breakfast 3 | Open-pollinated | Suttons seeds, UK |
| *R. sativus* | cv. Mino Early (Mooli) | Open-pollinated | Suttons seeds, UK |
| **Tomato** | | | |
| *Solanum lycopersicum* | cv. Moneymaker | Self- or open-pollinated | Suttons seeds, UK |

Abbreviation: PI, Plant Introduction collection, North Central Regional Plant Introduction Station, Iowa, USA.

### 4.2.7 Pathogenicity and race typing assays

The bacterial strains were grown on King's B medium plates for 48 h at 28 °C prior to inoculations. Bacterial suspensions were prepared in sterile distilled water and the inoculum concentration adjusted to approximately $2\times10^8$ cfu/ml as described in Section 2.2.3 (Chapter 2). Suspensions were filtered with a sterile muslin gauze to remove bacterial clumps that could block the spray heads.

Inoculations were carried out on four- to five-week-old plants and using two inoculation methods, spraying of entire plants and wounding of leaf veins and petioles. Spray inoculations were carried out following the method described by

Vicente *et al.* (2006). Plants were sprayed with bacterial suspensions using a DeVilbiss Atomizer 15 (DeVilbiss Health Care) connected to a pump (Huvema) at a pressure of 25 psi. The nozzle was kept at least 15 cm away from plants to avoid leaf damage. Plants were then kept inside a plastic bag for 24 h. Wound inoculations were carried out in three to four young leaves per plant and using one of two inoculation methods. One method consisted of piercing few sites in the leaf midrib and petiole of young leaves using a sterile pin loaded with bacteria directly collected from the medium agar plate as described by Vicente *et al.* (2006) for inoculation of *Xcr* strains. Leaf veins around the margins were also pierced for detection of V-shaped lesions caused by *Xcc* or dark and elongated but confined lesions caused by *Xcr*. The other method consisted of piercing leaf veins in several sites around the leaf margins using sterile mouth tooth forceps wrapped with cotton imbibed with a bacterial suspension, as described by Vicente *et al.* (2001) for inoculation of *Xcc* strains. A schematic representation of the inoculations sites in leaves, selected for both methods, is given in Figure 17.

After inoculation, all plants were kept in a glasshouse at 20 to 22 °C under a photoperiod of 10 h. Plants were examined regularly and symptoms were recorded at 14 and 21 days after inoculation.

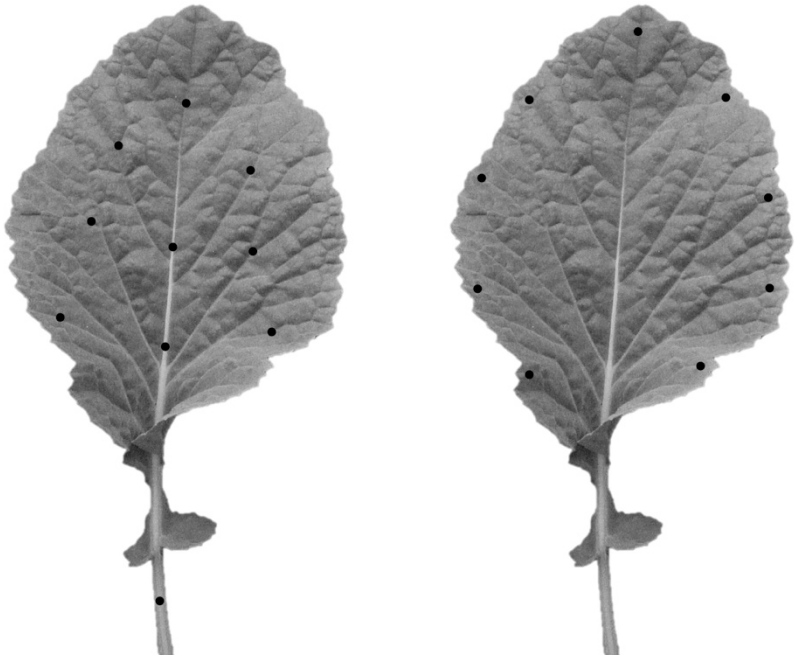


**Figure 17**. Schematic representation of selected sites in a leaf for wound inoculation using a pin (left) and forceps (right)

Pathogenicity tests were carried out in three main experiments as follows:

1) Preliminary pathogenicity tests of 11 *Xanthomonas* and *Pseudomonas* strains isolated from cauliflower leaf samples collected in Mauritius in 2012, were carried out in five plant lines: cauliflower cv. Local (the same cultivar from which strains were isolated), three *Brassica* differentials of *Xcr* and *Xcc* races (cauliflower cv. Miracle, Savoy cabbage cv. Wirosa, oilseed rape line COB60) and tomato cv. Moneymaker (susceptible control for *Xcr*). All strains were tested by wound inoculation using the pin method, as a quick method to identify pathogenic strains and discriminate between the *Xcc* and *Xcr* (Vicente *et al*., 2006). Spray inoculations were also carried out with two *X. campestris* strains (HRI8821 and 8823) in cauliflower cv. Local, and all *Pseudomonas* strains in the five plant lines mentioned above. The reference strain of *Xcc* race 4 HRI1279A was used as a control.

2) Race typing of a selection of nine *X. campestris* strains from Mauritius, either isolated in this study (HRI8821 and 8823) or isolated previously (HRI8506, 8507, 8514 and 8516A), and from South Carolina (HRI8806, 8807 and 8808), was carried out in ten plant lines that included *Brassicaceae* differentials of *Xcc* and *Xcr* races and tomato. Inoculations were carried out by spray and wound using the forceps method. The reference strains of *Xcc* races 4, 5 and 6 and *Xcr* race 2 (Table 21) were used as controls.

3) Race typing of the newly received pathotype strain of *Xcr* (HRI8803, NCPPB1946) and five other strains received as *Xcr* of unknown race (HRI7803, 8474, 8834, 8835 and 8804), was carried out in up to 11 plant lines that included *Brassicaceae* differentials of *Xcc* and *Xcr* races and tomato. Inoculations were carried out by spray and wound using the pin method. For comparison, the reference strain of *Xcr* races 1, 2 and 3, and the *Xcc* strain HRI3883, were used. The latter strain was also included in the experiment because it was deposited in the HRI collection as a clone of the pathotype strain of *Xcr* received from the NCPPB collection (strain NCPPB1946), but it has been identified as *Xcc* race 5 (Vicente *et al.*, 2001; Vicente *et al.*, 2006).

In all pathogenicity tests, two plants of each line were tested with each strain and inoculation method.

## 4.1 RESULTS

### 4.1.1 Bacterial strains isolated from field-grown cauliflowers from Mauritius

A total of 11 bacterial strains were isolated from leaf samples collected from cauliflowers (cv. Local) grown in the field in Mauritius in 2012 (Table 20). Although samples arrived dried in our laboratory, spots and enlarged necrotic lesions were easily identified as shown in Figure 18.

**Figure 18**. Cauliflower leaf samples received from Mauritius in October 2012 and used for isolation of bacteria.

On King's B medium (King *et al.*, 1954), only four strains were fluorescent (HRI8822B, 8824B, 8827B and 8827C) and seven were non-fluorescent (HRI 8821, 8822A, 8823, 8824A, 8825, 8826A and 8827A). The latter strains produced yellow-pigmented colonies on YDC medium (Figure 19), a feature that is typical, but not exclusive, of *Xanthomonas* bacteria (Schaad *et al.*, 2001; Saddler & Bradbury, 2005).

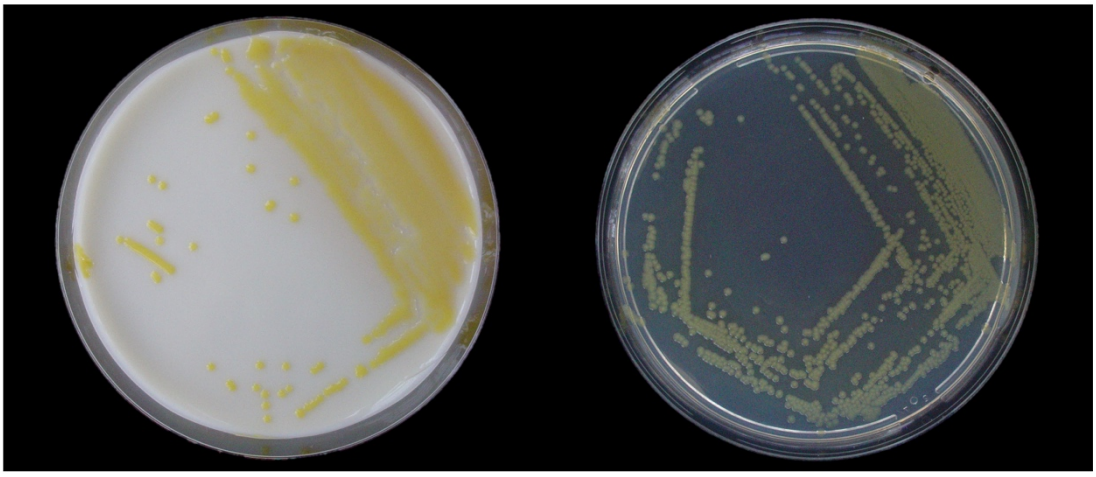


**Figure 19**. Bacterial colonies of strain HRI8821 grown on yeast dextrose calcium carbonate agar medium (left) and King's B medium (right).

### 4.1.2 Characterization of bacterial strains based on *16S* gene sequences

Partial sequences of the *16S* gene (1331 to 1382 nt long) were obtained from strains isolated from *Brassicas* spp. with leaf spot and blight symptoms grown in Mauritius and South Carolina, namely: 11 fluorescent and non-fluorescent strains isolated in this study from leaf samples collected in Mauritius in 2012 (described in the Section above), four strains isolated from leaf samples collected in Mauritius in 2009, and three strains from South Carolina that have been identified as *Xcc* (Wechter *et al.*, 2008) (strains listed in Table 20). Additionally, *16S* sequences were also obtained from reference strains of *Xcc* races 4, 5 and 6 (HRI1279A, 3880 and 6181) and *Xcr* race 2 (HRI8305). All sequences obtained in this study are listed in Appendix 10.

In BLASTN searches of *16S* gene sequences from Mauritian strains against the nucleotide database of NCBI, six non-fluorescent strains isolated in 2012 (HRI8821,

8823, 8824A, 8825, 8826A and 8827A) and the four strains isolated in 2009 (HRI8506, 8507, 8514 and 8516A) had equally high score hits with five *Xanthomonas* species (*arboricola*, *campestris*, *cynarae*, *gardeneri* and *hortorum*; sequence identity 100% and query coverage ≥ 99.9%) (Appendix 11). The remaining strains (the non-fluorescent strain HRI8822A and the fluorescent strains HRI8822B, 8824B, 8827B and 8827C) had the highest score hit with *Pseudomonas* species (sequence identity and query coverage ≥ 99.9%) (Appendix 11). Strains HRI8822B and 8824B had the best hit with *P. cichorii*, strains HRI8827B and 8827C with *P. viridiflava* and strain HRI8822A with *P. argentinensis*.

The *Xanthomonas* strains from Mauritius (isolated in 2009 and 2012) differed from strains from South Carolina by one nucleotide change in the *16S* gene sequences obtained, and no differences were observed among strains within each of these groups. The phylogenetic tree based *16S* gene sequences of *Xanthomonas* strains from Mauritius and South Carolina, reference strains of *Xcc* and *Xcr*, and the type strains of all currently validly named *Xanthomonas* species (listed in Appendix 10), showed an indistinct grouping of the Mauritian strains and strains of several *Xanthomonas* species, and the strains from South Carolina grouped with the reference strain of *Xcc* race 6 with low bootstrap support (65%) (Appendix 12).

The phylogenetic analysis of *16S* gene sequences from the *Pseudomonas* strains isolated in this study and reference strains of species of the main *Pseudomonas* phylogenetic groups (Anzai *et al.*, 2000), species identified in the 100 top hits of BLASTN searches and *Pseudomonas* pathogens of crucifers (*P. cichorii*, *P. syringae* pv. *maculicola*, *P. cannabina* pv. *alisalensis*), further supported that strains isolated in this study belong to three different *Pseudomonas* species (Figure 20). Strains HRI8822B and 8824B grouped with *P. cichorii*, strains HRI8827B and 8827C grouped with *P. viridiflava*, and strain HRI8822A fell within the *P. argentinensis* group. All these groups were supported by high bootstrap values (≥ 88%). All sequences used in this analysis are listed in Appendix 10.

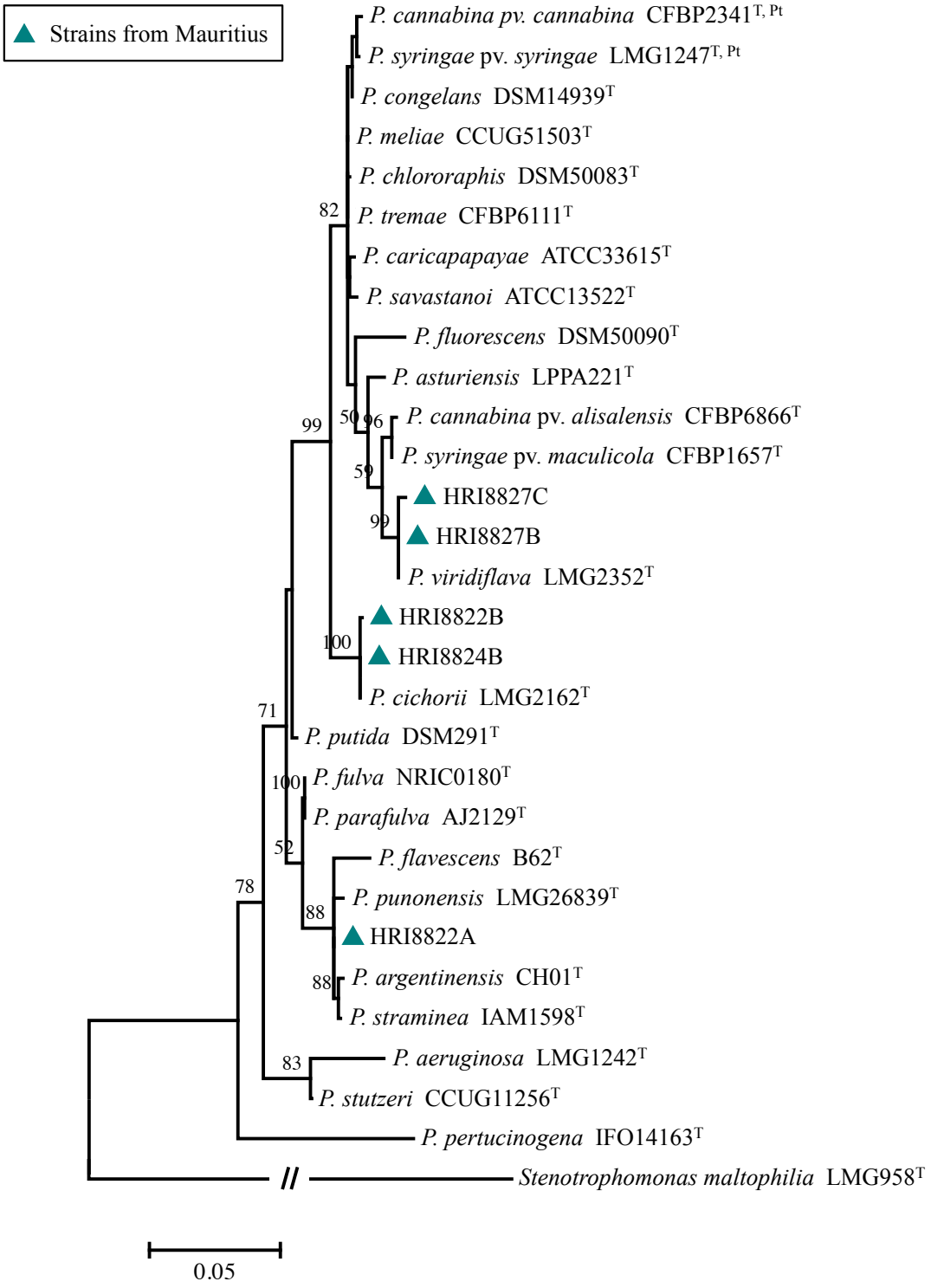



**Figure 20**. Phylogenetic tree based on *16S* gene partial sequences of *Pseudomonas* strains isolated from cauliflowers grown in Mauritius and reference strains of *Pseudomonas* species and pathovars. The tree was constructed by using the Maximum Likelihood method (Felsenstein, 1981) based on the Kimura 2-parameter model (Kimura, 1980) and it was rooted on *S. maltophilia*. Bootstrap values ≥ 50% from 1000 replicates are shown above the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 30 nucleotide sequences and 1297 nucleotide positions, and it was conducted in MEGA6 software (Tamura *et al*., 2013). Strain accessions are indicated. T indicates species type strains.

### 4.1.3 Characterization of *Xanthomonas* strains based on *gyrB* gene sequences

Partial *gyrB* gene sequences (542 to 820 nt long) were obtained from *Xanthomonas* strains isolated from *Brassicas* spp. grown in Mauritius (a selection of three strains isolated in 2012, HRI8821, 8823 and 8825; and strains isolated in 2009, HRI8506, 8507, 8514 and 8516A) and South Carolina (strains HRI8806, 8807 and 8808), as well as from reference strains of *Xcc* races 4, 5 and 6 (HRI1279A, 3880 and 6181) and *Xcr* race 2 (HRI8305) (Appendix 10). The strains from Mauritius differed by one nucleotide change: strains HRI8821, 8823, 8825 and 8514 shared a mutation not present in strains HRI8506, 8507 and 8516A. Regarding the strains from South Carolina, no differences were identified in their sequences and they shared 100% sequence identity with the Mauritian strains HRI8506, 8507 and 8516A.

In the phylogenetic tree based on partial *gyrB* sequences of strains from Mauritius and South Carolina and representative strains of all validly named *Xanthomonas* species and *X. campestris* pathovars and races (listed in Appendix 10), all *Xanthomonas* strains from Mauritius and South Carolina grouped with strains of *X. campestris* with high bootstrap support (82%) (Figure 21). These results confirmed that strains from Mauritius and South Carolina belong to the species *X. campestris*. Although no clear separation was observed between *X. campestris* pathovars in the phylogenetic tree, the strains from Mauritius and South Carolina shared the highest sequence identity with *Xcc* strains (≥ 99.6%; except for *Xcc* race 5) and lower sequence identity with the *Xcc* race 5 strain (≤ 99.2%) and strains of the remaining *X. campestris* pathovars (≤ 99.4%).

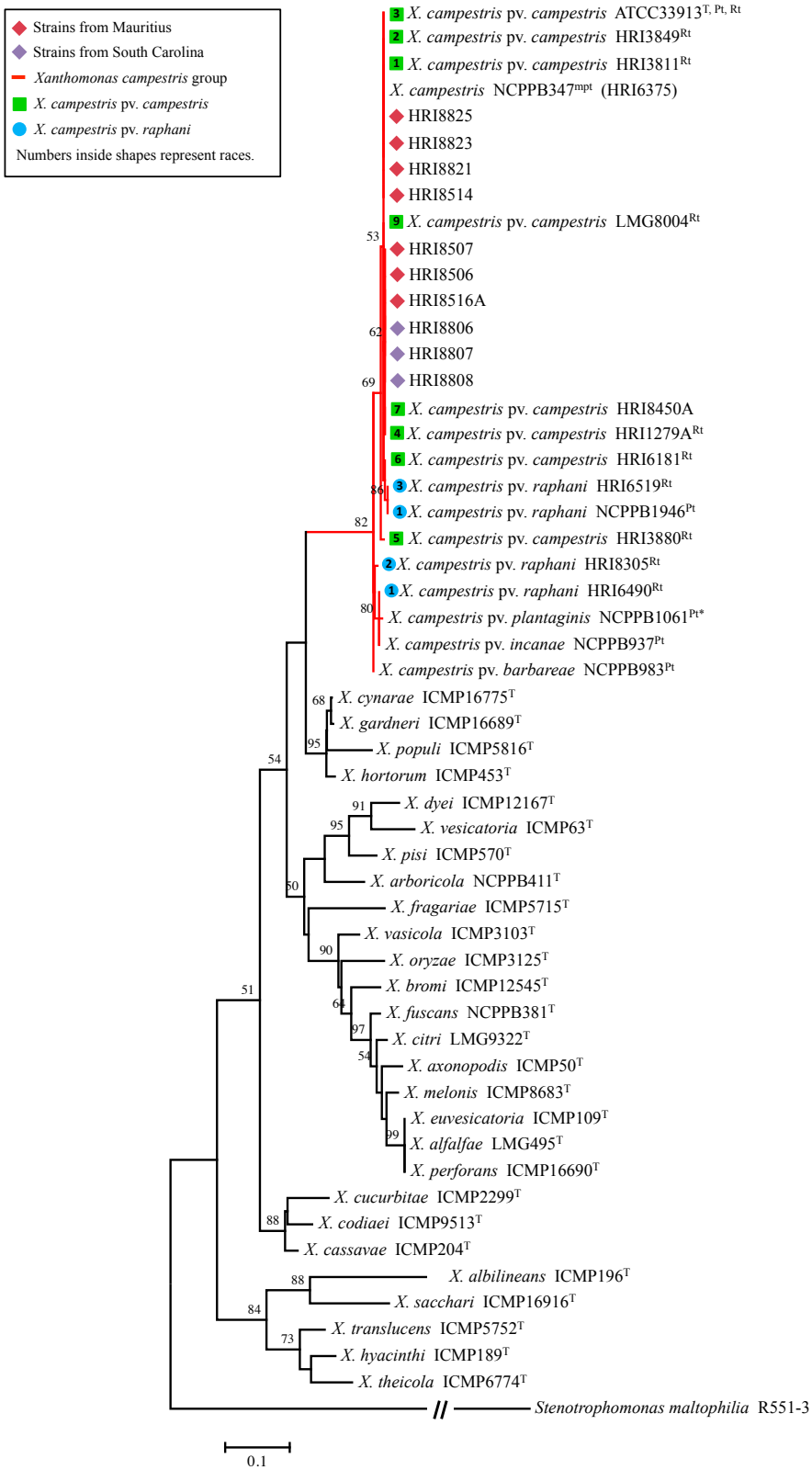



**Figure 21**. (Previous page) Phylogenetic tree based on *gyrB* gene partial sequences of *Xanthomonas campestris* strains isolated from brassicas grown in Mauritius and South Carolina (USA) and reference strains of *Xanthomonas* species and *X. campestris* pathovars. The tree was constructed by using the Maximum Likelihood method (Felsenstein, 1981) based on the Tamura-Nei model (Tamura & Nei, 1993) and it was rooted on *S. maltophilia*. Bootstrap values ≥ 50% from 1000 replicates are shown above the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 54 nucleotide sequences and 500 nucleotide positions, and it was conducted in MEGA6 software (Tamura *et al.*, 2013). Strain accessions are indicated. Abbreviations: mpt, misidentified pathotype of *X. campestris* pv. *armoraciae* (Vicente *et al.*, 2006; Fargier & Manceau, 2007); Pt, pathotype strain; Rt, race-type strain; T, species-type strain.

### 4.1.4 Pathogenicity of bacterial strains isolated from leaves of cauliflowers grown in Mauritius in 2012

The results of preliminary pathogenicity tests carried out with all *Xanthomonas* and *Pseudomonas* strains isolated in this study from leaf samples of cauliflower cv. Local grown in the field in Mauritius (in 2012), are summarized in Table 24.

**Table 24**. Interactions between *Xanthomonas* and *Pseudomonas* strains from Mauritius and *Brassica* spp. and tomato plants [a]

| | HRI strain accession | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Xanthomonas* | | | | | | | *Pseudomonas* | | | | |
| **Plant description** | 8821 | 8823 | 8824A | 8825 | 8826A | 8827A | 1279A[b] | 8822A | 8822B | 8824B | 8827B | 8827C |
| Cauliflower cv. Local | + | + | + | + | + | + | + | − | + | + | − | − |
| Cauliflower cv. Miracle | + | + | + | + | + | + | + | − | + | − | − | − |
| Savoy cabbage cv. Wirosa | + | + | + | + | + | + | + | − | + | − | − | − |
| Oilseed rape line COB60 | − | − | − | − | − | − | − | − | + | − | − | − |
| Tomato cv. Moneymaker | − | − | − | − | − | − | − | − | + | + | − | − |

Symbols and abbreviation: +, compatible interaction (susceptibility, highlighted in yellow); −, incompatible interaction (resistance, highlighted in green); (+), weakly pathogenic; HRI, formerly Horticulture Research International, now University of Warwick Crop Centre, Wellesbourne, UK.

[a] The interactions shown for *Xanthomonas* strains were assessed in wound-inoculated plants except for interactions between strains HRI8821, 8823 and 1279A and cauliflower cv. Local that were assessed in wound- and spray-inoculated plants. The interactions shown for *Pseudomonas* strains were assessed in spray-inoculated plants. The inoculation methods used are described in Section 4.2.7 of the present Chapter. Two plants were tested with each strain and inoculation method. Symptoms were recorded 14 to 21 days after inoculation.

[b] Type strain of *Xanthomonas campestris* pv. *campestris* race 4 used as a control.

All six *Xanthomonas* strains tested were pathogenic on cauliflower cv. Local, cauliflower cv. Miracle and Savoy cabbage cv. Wirosa, and did not produce symptoms in oilseed rape line COB60 and tomato cv. Moneymaker (Table 24). Wound inoculated leaves showed necrotic lesions that started at the inoculation sites and rapidly expanded into large areas of the leaves resembling blight (Figures 22a,b). In some cases, wounded leaves dried out and dropped (Figure 22c). Often, lesions were also observed in non-inoculated leaves and generally started in the lower half of the leaves, along the midrib and lateral veins (Figures 22c,d). In those leaves, chlorosis of tissues around the leaf veins was initially observed (Figure 22d) and rapidly progressed into darkening of veins and development of necrotic lesions.

These symptoms were observed from 14 to 21 days after inoculation. The strains HRI8821 and 8823 were also tested by spray inoculation in the cauliflower cv. Local (cultivar from which strains were isolated), and produced small dark leaf spots and large necrotic lesions. The type strain of *Xcc* race 4 strain (HRI1279A) used as a control in wound and spray inoculations, produced similar symptoms, but the development of spots and leaf blight was less prominent. None of the *Xanthomonas* strains tested caused black and sunken lesions in the leaf midrib and petioles, which are typically caused by *Xcr* upon wound inoculation (Vicente *et al*., 2006).

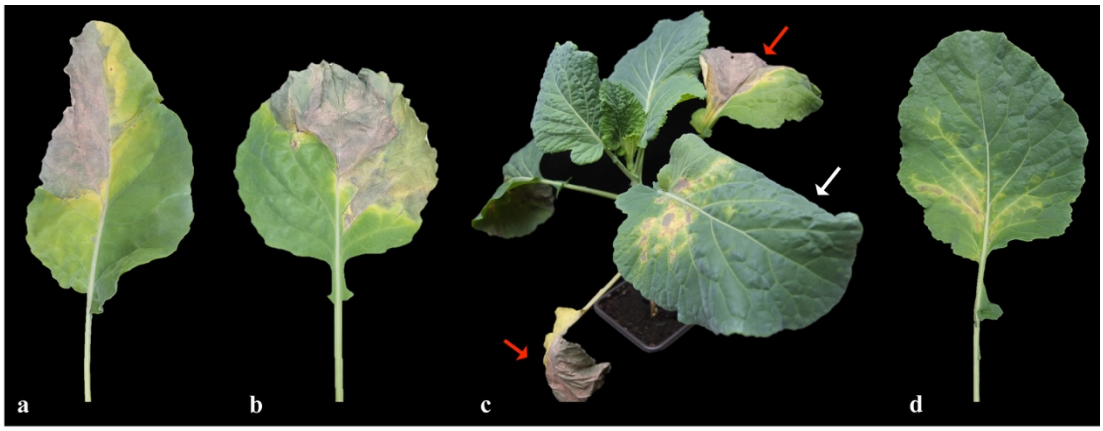



**Figure 22**. Symptoms induced on brassicas by *Xanthomonas* strains from Mauritius, 21 days after wound inoculation. **a**, necrotic lesion induced on a wounded leaf of cauliflower cv. Miracle by strain HRI8821; **b**, necrotic lesions induced on a wounded leaf of Savoy cabbage cv. Wirosa by strain HRI8821; **c**, systemic infection of a Savoy cabbage cv. Wirosa plant with lesions occurring in a non-inoculated leaf (white arrow) and inoculated leaves (red arrows) induced by strain HRI8827A; **d**, chlorosis of veins in a non-inoculated leaf of Savoy cabbage cv. Wirosa induced by strain HRI8824A.

*Pseudomonas* strains did not produce symptoms in any plant inoculated using the wound method, but two strains (HRI8822B and 8824B) produced symptoms in sprayed plants (Table 24). Strain HRI8822B was pathogenic on all plants tested whereas strain 8824B was only pathogenic on cauliflower cv. Local and tomato. Both strains caused small dark leaf spots with irregular margins and dark lesions in stems and leaf petioles that were visible five to seven days after inoculation (Figures 23a,b,d). Development of large necrotic lesions in leaves was also observed (Figure 23c).


**Figure 23**. Symptoms induced on brassica and tomato plants by the *Pseudomonas* strain HRI8822B, five to seven days after spray inoculation. **a**, leaf, and **b**, stem and petiole, of cauliflower cv. Miracle; **c**, leaf of oilseed rape line COB60; and **d**, leaf of tomato cv. Moneymaker leaf.

### 4.1.5 Race typing of *Xanthomonas campestris* strains associated with outbreaks of a leaf spot and blight disease of brassicas in Mauritius and South Carolina

A summary of the results obtained in pathogenicity tests for race typing of a selection of six *X. campestris* strains from Mauritius (isolated in 2009 and 2012) and three *Xcc* strains from South Carolina, is presented in Table 25. In general, symptoms became clearly visible between 10 to 14 days after inoculation and the results obtained by wound and spray inoculation methods were congruent.

All six *X. campestris* strains from Mauritius showed interactions with all plants tested as those observed with the type strain of *Xcc* race 4 (HRI1279A). They were pathogenic on Savoy cabbage cv. Wirosa, cauliflower cv. Miracle, *B. oleracea* line SxD1 and radish cv. Mino Early, but not pathogenic on any of the remaining plants tested (Table 25). In turn, strains from South Carolina were pathogenic on all plants tested except tomato and produced the same pattern of reactions as observed with the type strain of *Xcc* race 6 (HRI6181) (Table 25). However, they seemed to be more aggressive to mustard line FBLM2 than the type strain of *Xcc* race 6 (HRI6181).

**Table 25**. Interactions between *Xanthomonas campestris* strains from Mauritius and South Carolina and plant differentials of races of *Xanthomonas campestris* pathovars *campestris* and *raphani* [a]

| | HRI strain accession | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Xc* from Mauritius | | | | | | *Xcc* r4[b] | *Xc* from South Carolina | | | *Xcc* r6[b] | *Xcc* r5[b] | *Xcr* r2[b] |
| **Plant cultivar/line** | **8821** | **8823** | **8506** | **8507** | **8514** | **8516A** | **1279A$^{Rt}$** | **8806** | **8807** | **8808** | **6181$^{Rt}$** | **3880$^{Rt}$** | **8305$^{Rt}$** |
| Savoy cabbage cv. Wirosa | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Cauliflower cv. Miracle | + | + | + | + | + | + | + | + | + | + | + | − | − |
| *B. oleracea* line SxD1 | + | + | + | + | + | + | + | + | + | + | + | − | nt |
| Oilseed rape line COB60 | − | − | − | − | − | − | − | + | + | + | + | + | + |
| Mustard line FLBM2 | − | − | − | − | − | − | − | + | + | + | (+) | (+) | + |
| Ethiopian mustard line PIC1 | − | − | − | − | − | − | − | + | + | + | + | + | + |
| Turnip cv. Just Right | − | − | − | − | − | − | − | + | + | + | + | + | + |
| Turnip cv. Seven Top | +/− | − | +/− | +/− | +/− | +/− | +/− | + | + | + | + | +/− | nt |
| Radish cv. Mino Early | + | + | + | + | + | + | + | + | + | + | + | − | − |
| Tomato cv. Moneymaker | − | − | − | − | − | − | − | − | − | − | − | − | + |

Symbols and abbreviations: +, compatible interaction (susceptibility, highlighted in yellow); −, incompatible interaction (resistance, highlighted in green); (+), weakly pathogenic; +/–, variable reaction; HRI, formerly Horticulture Research International, now University of Warwick Crop Centre, Wellesbourne, UK; nt, not tested; r, race; *Xc*, *Xanthomonas campestris*; *Xcc*, *X. campestris* pv. *campestris*; *Xcr*, *X. campestris* pv. *raphani*.

[a] Plants were tested by spray and wound inoculation as described in Section 4.2.7 of the present Chapter. Two plants were tested with each strain and inoculation method. Symptoms were recorded 14 to 21 days after inoculation. The combined results of both methods are presented.

[b] Race-type strains used as controls.

Leaf blight was the most common symptom induced by strains from Mauritius and South Carolina in wound inoculated plants. Leaf tissues became necrotic and brittle and lesions expanded into V-shaped or irregular lesions with a clear defined border between the dark necrotic tissues and the green to slightly chlorotic tissues (Figure 24). Necrosis of leaf tissues was also observed in plants inoculated with the type strains of *Xcc* races 4, 5 and 6, but it was less severe than in plants inoculated with strains from Mauritius and South Carolina (Figure 24).

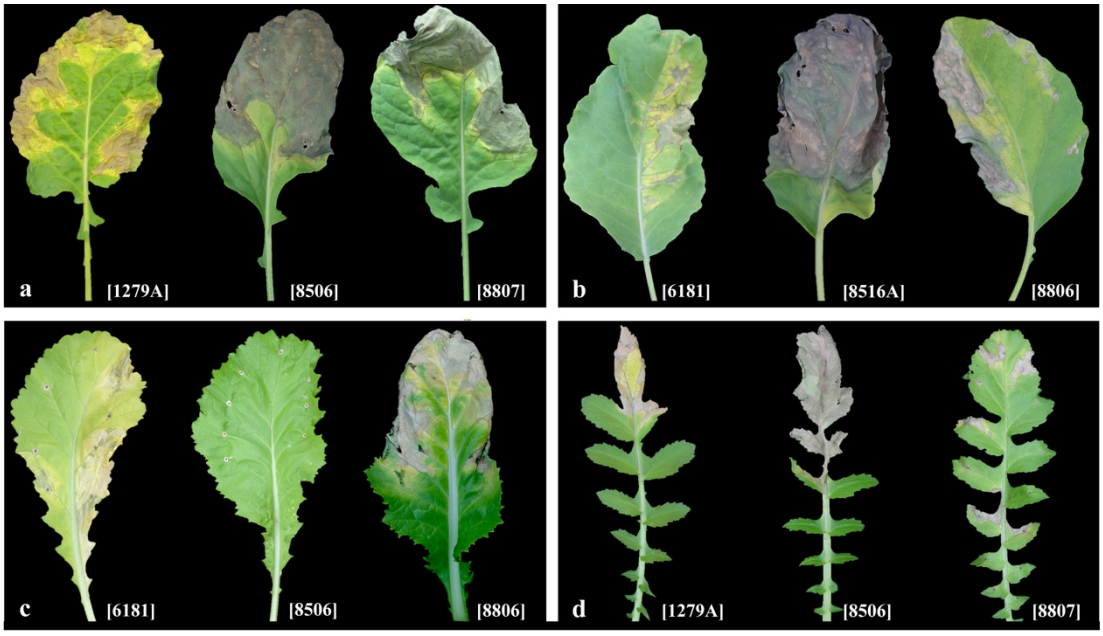



**Figure 24**. Symptoms induced on leaves from four host cultivars 21 days after wound inoculation with a *Xanthomonas campestris* pv. *campestris* strain (HRI1279A or 6181, left in each panel) or a *X. campestris* strain from either Mauritius (HRI8506 or 8516A, centre) or South Carolina (HRI8806 or 8807, right). The host cultivars include: **a**, Savoy cabbage cv. Wirosa; **b**, cauliflower cv. Miracle; **c**, mustard cv. FBLM2; and d, radish cv. Mino Early.

In spray-inoculated plants, strains from Mauritius and South Carolina as well as type strains of *Xcc* races 4, 5 and 6, generally induced V-shaped and irregular chlorotic/necrotic lesions in leaves and blackening of leaf veins (Figure 25). Small dark leaf spots were also observed in several compatible interactions, but spots occurred in minor numbers in most cases. The development of small leaf spots with chlorotic haloes was particularly prominent in cauliflower cv. Miracle and Savoy cabbage cv. Wirosa inoculated with strains from Mauritius (Figures 25a,b). Small leaf spots and dark leaf veins were clearly visible approximately 10 days after inoculation and rapidly progressed into larger necrotic lesions in the next following days (Figure 26). These symptoms were distinct from the symptoms caused by the *Xcr* strain (used as a control) that were characterized by well-defined necrotic leaf spots and dark sunken lesions in petioles.

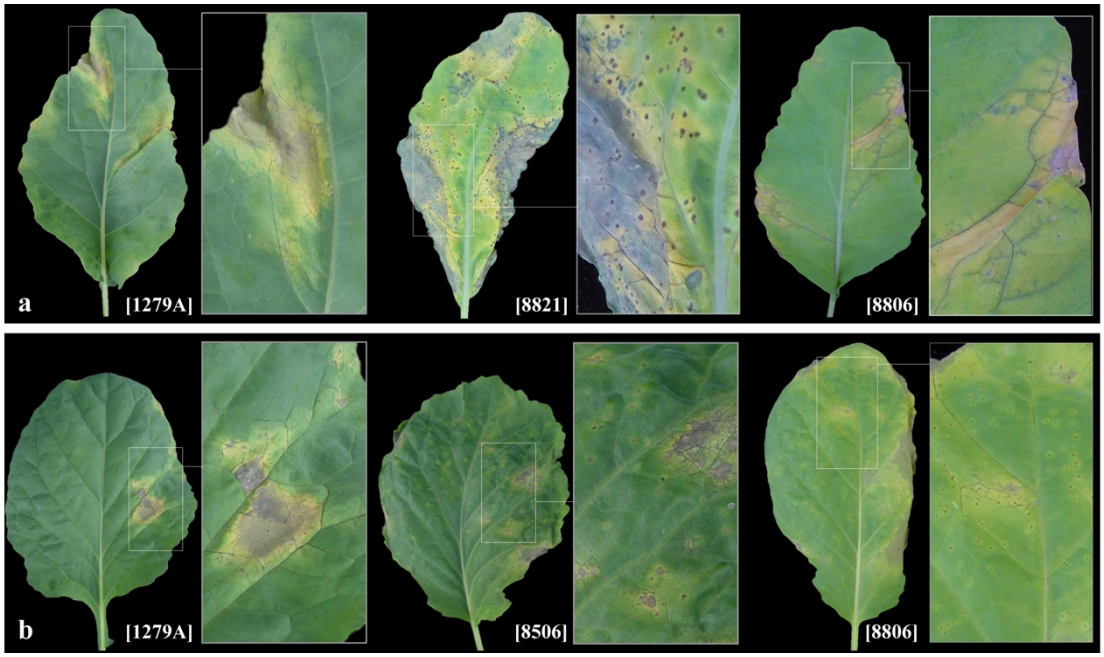



**Figure 25**. Symptoms induced on leaves of two *Brassica oleracea* cultivars 14 days after spray inoculation with a strain of *Xanthomonas campestris* pv. *campestris* (HRI1279A, left in panels) or *X. campestris* strains from Mauritius (HRI8821 or 8506, centre) and South Carolina (HRI8806, right). The host cultivars include: **a**, cauliflower cv. Miracle, and **b**, Savoy cabbage cv. Wirosa. White rectangles indicate patches of infected tissue magnified approximately 3×.

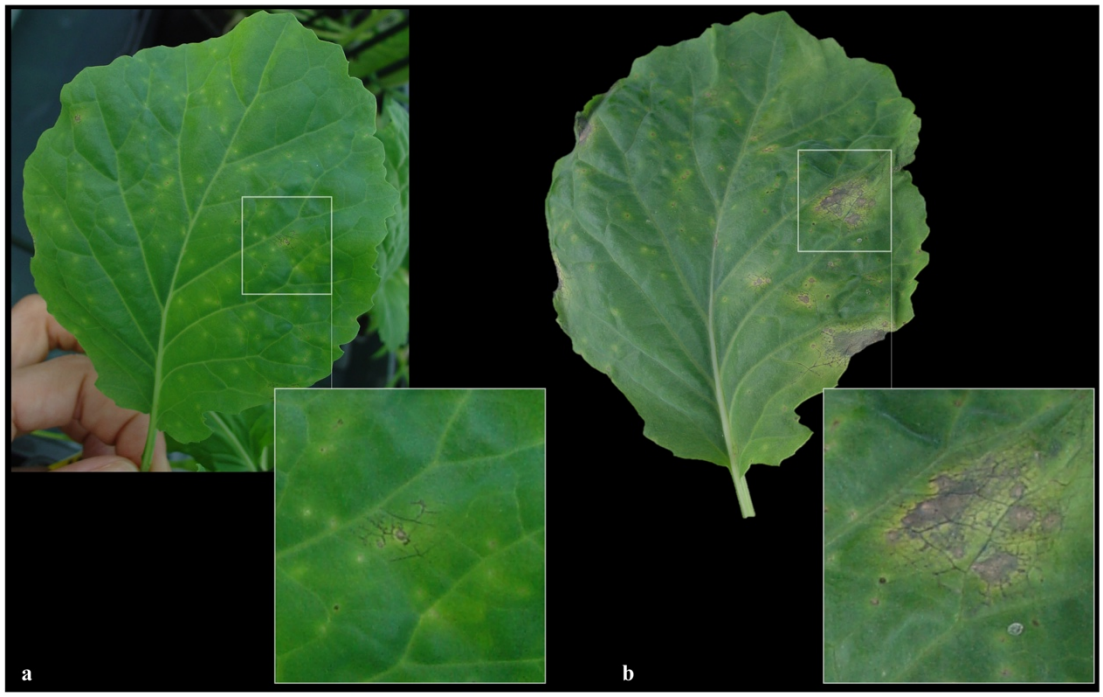



**Figure 26**. Development of leaf spots, darkened veins and necrotic lesions on leaves of Savoy cabbage cv. Wirosa, following spray inoculation with a *Xanthomonas campestris* strain from Mauritius (HRI8506) at: **a**, 10 days; and **b**, 14 days after inoculation. White rectangles indicate patches of infected tissue magnified approximately 3×.

### 4.1.6 Pathogenicity of strains received as *Xanthomonas campestris* pv. *raphani*

The results obtained in pathogenicity and race typing assays of six strains received as *Xcr* (HRI8803, 7803, 8474, 8834, 8835 and 8804) are summarized in Table 26.

**Table 26**. Pathogenicity of *Xanthomonas campestris* strains in plant differentials of races of *Xanthomonas campestris* pathovars *campestris* and *raphani* [a]

| | HRI strain accession | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Xcr* | | | | | *Xcr* r1 | *Xcr* r2 | *Xcr* r3 | *Xcc* | *Xcc* r5 |
| **Plant cultivar/line** | **8803** [Pt] | **7803** | **8474** | **8834** | **8835** | **6490** [Rt, b] | **8305** [Rt, b] | **6519** [Rt, b] | **8804** | **3883** [b] |
| Savoy cabbage cv. Wirosa | ls+ | ls+ | ls+ | ls+ | ls+ | ls+ | ls+ | ls+ | va+ | va+ |
| Cauliflower cv. Miracle | ls+ | – | – | ls+ | ls+ | ls+ | – | ls+ | va+ | – |
| *B. oleracea* line SxD1 | nt | nt | nt | nt | nt | nt | nt | nt | va+ | nt |
| Oilseed rape line COB60 | – | ls+ | ls(+) | ls+ | ls+ | – | ls+ | ls(+)/+ | va+ | va+ |
| Mustard line FBLM2 | – | ls+ | ls+ | ls+ | ls+ | – | ls+ | ls+ | – | va(+) |
| Ethiopian mustard line PIC1 | ls+ | ls+ | ls+ | ls(+) | ls(+) | ls+ | ls+ | ls(+)/+ | – | va(+) |
| Turnip cv. Just Right | – | ls+ | ls+ | ls+ | ls+ | – | ls+ | ls+ | va+ | va+ |
| Turnip cv. Seven Top | nt | nt | nt | nt | nt | nt | nt | nt | va+ | nt |
| Radish cv. Mino Early | ls+ | – | – | ls+ | ls+ | ls+ | – | ls+ | – | – |
| Radish cv. French Breakfast 3 | nt | nt | nt | ls+ | ls+ | ls+ | ls+ | ls+ | nt | nt |
| Tomato cv. Moneymaker | ls+ | ls+ | ls+ | ls+ | ls+ | ls+ | ls+ | ls+ | – | – |

Symbols and abbreviations: +, compatible interaction (susceptibility, highlighted in yellow); –, incompatible interaction (resistance, highlighted in green); (+), weakly pathogenic; HRI, formerly Horticulture Research International, now University of Warwick Crop Centre, Wellesbourne, UK; ls, leaf spot symptoms; nt, not tested; Pt, pathotype strain; r, race; Rt, race-type strain; va, vascular infection; *Xcc*, *X. campestris* pv. *campestris*; *Xcr*, *X. campestris* pv. *raphani.*

[a] Plants were tested by spray and wound inoculation as described in Section 4.2.7 of the present Chapter. Two plants were tested with each strain and inoculation method. Symptoms were recorded 14 to 21 days after inoculation. The combined results obtained with both inoculation methods are presented.

[b] Strains used as controls.

Five out of the six strains received as *Xcr*, namely HRI8803 (pathotype strain of *Xcr*, NCPPB1946), 7803, 8474, 8834 and 8835, induced typical leaf spot symptoms as those caused by the *Xcr* strains used as controls, following spray and wound inoculations of several plants (Table 26).

In spray-inoculated plants, *Xcr* strains caused necrotic leaf spots not limited by veins (Figures 27a-g) and dark sunken lesions in the leaf midrib and petioles (Figures 27d,h-j). Leaf spots were visible in both sides of the leaves and they were often surrounded by a chlorotic halo (Figures 27a,b). Spots were also observed on the surface of radish hypocotyls of cv. French Breakfast; these lesions were sunken, but not very deep (Figures 27k,l).

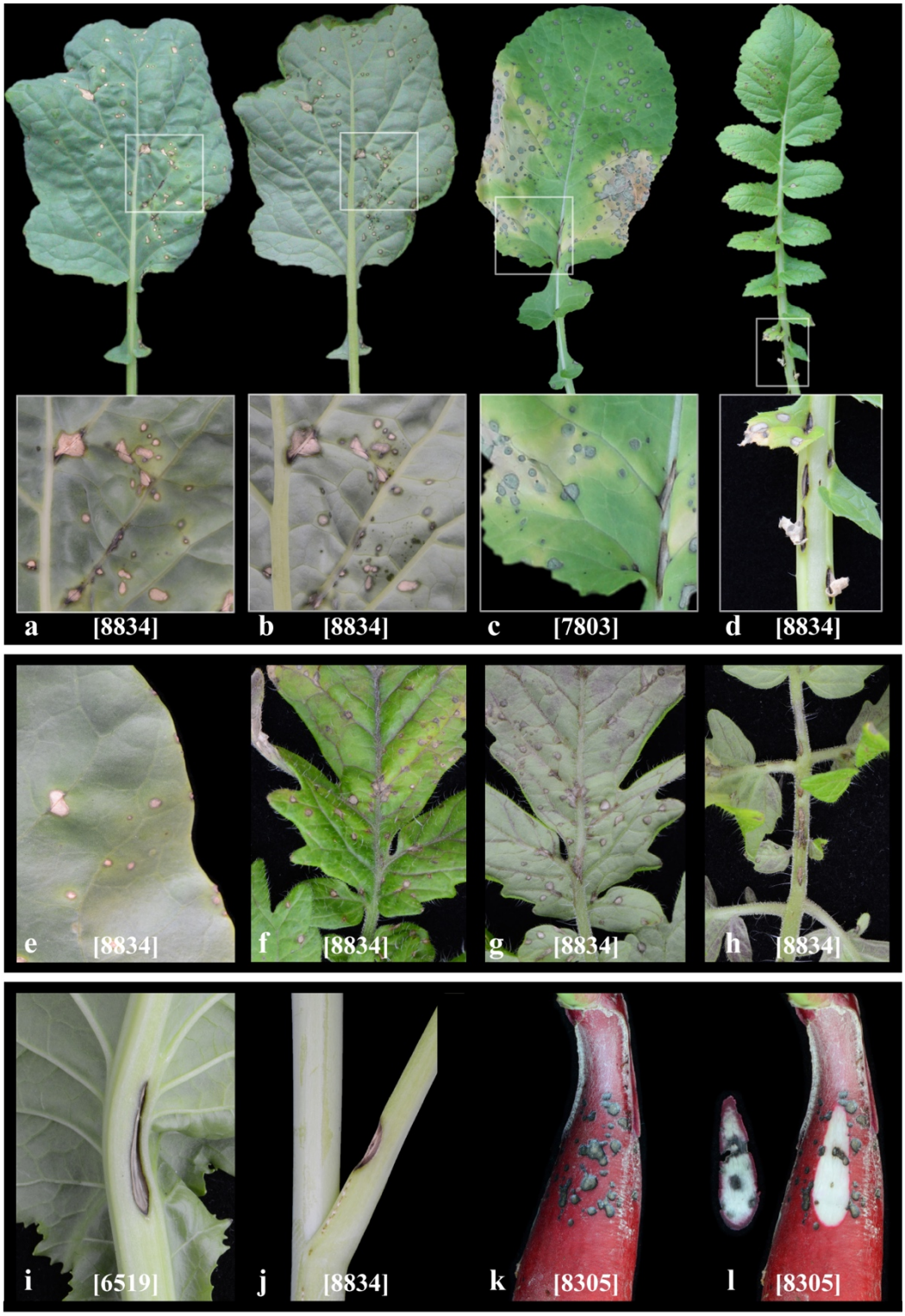



**Figure 27**. Symptoms induced by *Xanthomonas campestris* pv. *raphani* (strains HRI8834, 7803, 6519 and 8305) on brassicas, radish and tomato plants 21 days after spray inoculation. Host cultivars include: **a** and **b**, Savoy cabbage cv. Wirosa (leaf, upper side in a; leaf, lower side); **c**, Ethiopian mustard line PIC1 (leaf, upper side); **d**, radish cv. Mino Early (leaf, upper side); **e**, cauliflower cv. Miracle (leaf section; upper side); **f** to **h**, tomato cv. Moneymaker (leaf sections, upper side in **f**; lower side in **g** and **h**); **i**, mustard line FBLM2 (leaf midrib, lower side); **j**, cauliflower cv. Miracle (petiole); **k** and **l**, hypocotyl of radish cv. French Breakfast 3. White rectangles indicate patches of infected tissue magnified approximately 3×.

In wound inoculations, the *Xcr* strains caused localized necrosis of the tissues near the inoculation sites (Figures 28a-d). In rare cases, lesions progressed along the leaf veins for short distances (no more than a couple of cm) (Figure 28d). Lesions were markedly black and sunken in the leaf midrib and petioles (Figures 28a-c) and petiole girdling was commonly observed as shown in Figure 28c.

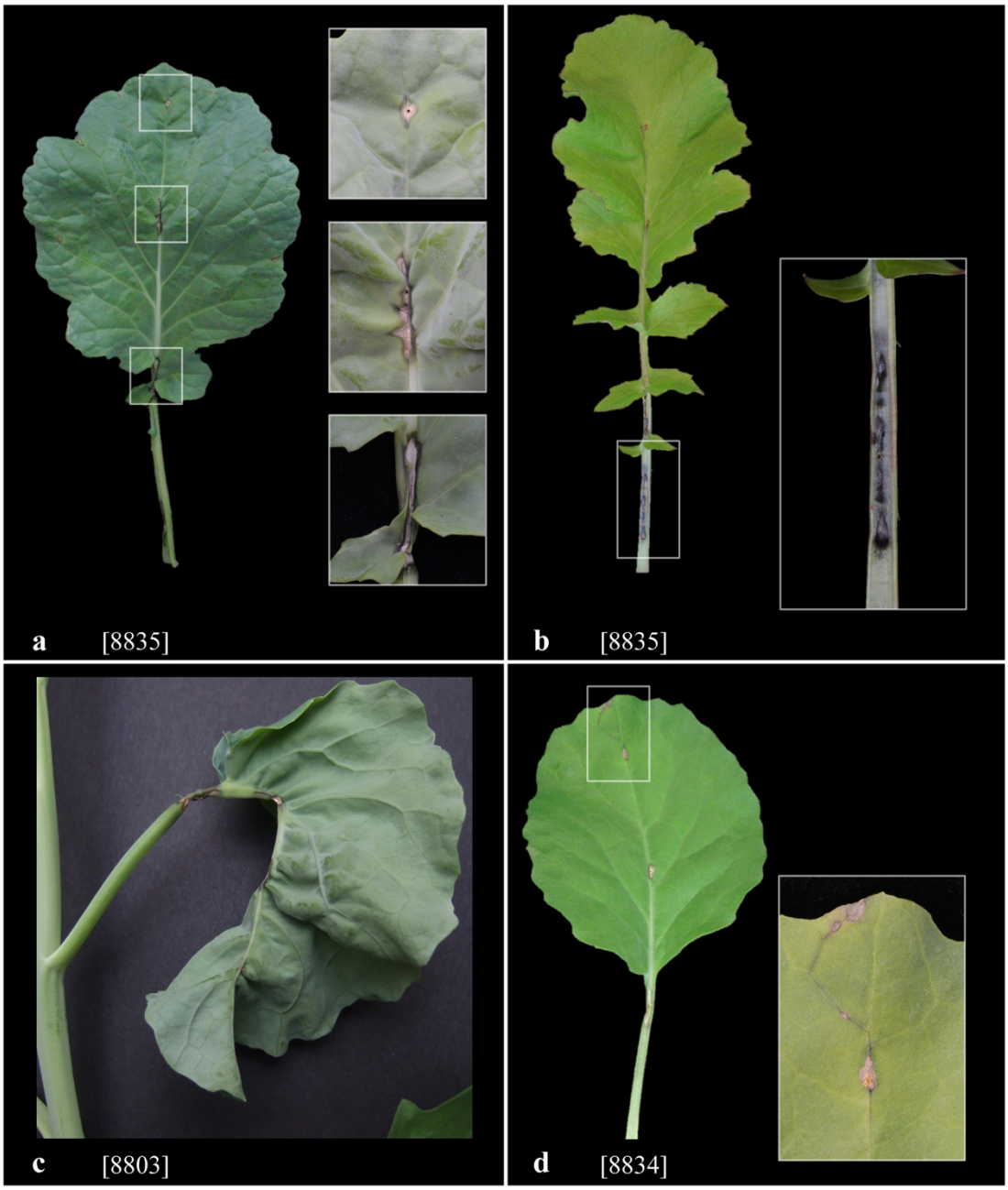



**Figure 28**. Symptoms induced by *Xanthomonas campestris* pv. *raphani* strains on wound inoculated leaves of brassicas and radish. Host cultivars include: **a**, Savoy cabbage cv. Wirosa (upper side; strain HRI8835; 14 days after inoculation, dai); **b**, radish cv. French Breakfast 3 (upper side; strain HRI8835; 14 dai); **c**, cauliflower cv. Miracle (lower side; strain HRI8803; 9 dai); **d**, Savoy cabbage cv. Wirosa (upper side; strain HRI8834; 14 dai); White rectangles indicate patches of infected tissue magnified approximately 3×.

In interactions between the *Xcr* strains and plant differentials tested, the pathotype strain of *Xcr* (HRI8803) was similar to *Xcr* race 1 (HRI6490), the strain isolated in the UK (HRI8474) and the strain from Africa (HRI7803) were similar to *Xcr* race 2 (HRI8305) although the strain HRI8474 was only weakly pathogenic on oilseed rape line COB60, and the strains from Italy (HRI8834 and 8835) were similar to *Xcr* race 3 (HRI6519) (Table 26).

The strain HRI8804 received from the NCPPB as *Xcr* (from India) caused V-shaped or irregular chlorotic/necrotic lesions and blackening of leaf veins, in sprayed and wound inoculated leaves, similar to the typical symptoms caused by *Xcc* (Figure 29). The development of leaf spots symptoms typical of *Xcr* was not observed. This strain produced reactions in the plant differentials (Table 26) similar to those described for *Xcc* race 1 (Vicente & Holub, 2013; Table 2, Chapter 1).

**Figure 29**. Symptoms induced by the *Xanthomonas campestris* pv. *campestris* strain HRI8804 on leaves of Savoy cabbage cv. Wirosa 20 days after inoculation by wounding (left) and spraying (right).

The strain HRI3883 that has been described by Vicente *et al.* (2001, 2006) as a clone of the pathotype strain of *Xcr* NCPPB1946, produced typical symptoms of *Xcc* as reported by those authors. However, the newly received clone of NCPPB1946 (strain HRI8803) caused typical leaf spots symptoms of *Xcr* and this results confirmed that the strain used by Vicente *et al.* (2001, 2006) has been mislabelled.

## 4.2 DISCUSSION

The main aim of this study was to characterize *X. campestris* bacteria associated with outbreaks of severe leaf spot and blight disease of cabbage and cauliflower that occurred in Mauritius (R. Lobin, pers. comm.), and compare them with *Xcc* strains associated with similar outbreaks reported in South Carolina (USA) (Wechter *et al.*, 2008). On the basis of a combination of molecular and pathology data presented in this Chapter, all *Xanthomonas* strains from Mauritius studied were identified as *Xcc* race 4 whilst strains from South Carolina were *Xcc* race 6. *Pseudomonas* sp. strains that can potentially have an effect in the development of disease symptoms were also identified.

Among bacterial strains from Mauritius isolated in two different years, 2009 (R. Lobin and J. Vicente, pers. comm.) and 2012 (this study), pathogenic *X. campestris* strains were identified in both years and two pathogenic fluorescent *Pseudomonas* sp. strains were identified in leaf samples collected in 2012.

*Xanthomonas* strains were identified based on molecular analysis of *16S* and *gyrB* gene sequences. The *16S* gene has been the most commonly used genetic marker to study bacterial phylogeny and taxonomy (Clarridge, 2004; Janda & Abbott, 2007). As a consequence, the number of *16S* gene sequences available in public databases greatly overtakes the number of sequences of any other bacterial genetic marker and include sequences from nearly all type strains of bacterial species (Yarza *et al.*, 2013). Comparisons of partial sequences of this gene with sequences available in the NCBI nucleotide database, confirmed that strains from Mauritius belong to the genus *Xanthomonas*. These strains had identical *16S* gene sequences and shared the highest sequence identity with several *Xanthomonas* species (*arboricola*, *campestris*, *cynarae*, *gardeneri* and *hortorum*). One mutation distinguished strains from Mauritius and South Carolina indicating that these strains are different.

As previously reported, the *16S* gene has a low resolution to differentiate *Xanthomonas* species (Hauben *et al.*, 1997) and other conserved genes including *gyrB* have been used to discriminate species and strains within species in the genus (Parkinson *et al.*, 2007; Young *et al.*, 2008; Parkinson *et al.*, 2009; Fargier *et al.*, 2011). Consequently, *gyrB* sequences from a large number of *Xanthomonas* strains

have been published including sequences of type strains of different species and pathovars. The *gyrB* gene sequences of strains from Mauritius and South Carolina allowed a clear identification to the species level confirming that these strains belong to *X. campestris*. Strains from Mauritius and South Carolina shared highest sequence identity with standard *Xcc* strains than with strains of other *X. campestris* pathovars. Differences among strains from Mauritius were detected independently of the year of isolation, but no differences were observed among strains from South Carolina. The latter strains may represent the same organism as it was also suggested by the analysis of repetitive sequence-based PCR genomic fingerprinting obtained for those strains by P. Wechter (pers. comm.).

In pathogenicity tests performed under glasshouse conditions, the strains from Mauritius and South Carolina caused vascular infections similar to infections caused by *Xcc* strains. Particularly in wound-inoculated plants, the development of symptoms in non-wounded leaves constituted a good evidence of the systemic movement of the pathogens throughout the vascular system from inoculated leaves to non-inoculated leaves. However, they seemed capable of causing a more severe necrosis of the leaf tissues when compared to the reference *Xcc* strains used in the pathogenicity tests. The mesophyll tissues rapidly collapsed and necrotic lesions expanded affecting large areas of the leaves with a clear boundary between diseased and healthy tissues and little chlorosis if any. Other *X. campestris* strains have been reported to cause similar blight symptoms (Yuen & Alvarez, 1985; Alvarez *et al.*, 1994; Shigaki, 1996; Alvarez, 2000) and have been considered to represent an aggressive variant of *Xcc* (Alvarez, 2000). The capability to induce blight seems to be variable within the *Xcc* population and some strains have been reported to cause stronger blight symptoms than others (Shigaki, 1996).

The development of leaf spots was prominent on cauliflower and Savoy cabbage plants sprayed with *Xanthomonas* strains from Mauritius. Leaf spots appeared very small and rapidly progressed into large and irregular necrotic lesions that were clearly different from leaf spots caused by *Xcr* strains which were generally confined to the site of infection. These observations confirmed that *Xanthomonas* strains from Mauritius were distinct from *Xcr*.

Strains from Mauritius were distinct from strains from South Carolina in their interactions with the plant differentials used to distinguish races of *Xcc* and *Xcr*

(Vicente *et al.*, 2006; Vicente & Holub, 2013; summarized in Table 2, Chapter 1). Strains from Mauritius (isolated from *B. oleracea* crops) were race 4, which is the race most commonly found in *B. oleracea* crops worldwide (Taylor *et al.*, 2002). In contrast, strains from South Carolina (isolated from *B. rapa* and *B. juncea*) were race 6, although they seemed to be more aggressive than the reference strain of *Xcc* race 6 (HRI6181) in mustard line FBLM2.

Among strains isolated in this study from samples collected in Mauritius, a few strains were identified as *Pseudomonas* sp. by analysis of *16S* gene sequences. Two strains (HRI8822B and 8824B) were found pathogenic at least on cauliflower cv. Local from Mauritius and tomato cv. Moneymaker. These strains might belong to the species *P. cichorii* because their *16S* gene sequences were 99-100% identical to the *16S* gene sequence of the type strain of this species and they grouped with this species in the *16S* phylogenetic tree. The phylogenetic analysis also confirmed that these strains were different from *P. syringae* pv. *maculicola* and *P. cannabina* pv. *alisalensis* that have been reported to cause leaf spot and blight diseases on crucifers (Zhao *et al.*, 2000b; Keinath *et al.*, 2006; Takikawa & Takahashi, 2014). *P. cichorii* has been previously reported to cause spots in leaves and stems in cabbage (Wehlburg, 1963) and it is possible that the pathogenic strains identified in this study belong to this species. However, because no other molecular and phenotypic traits were studied, a more accurate identification of these strains will require further characterization, for example by sequencing the *rpoB* gene that has been used for identification of strains within the *Pseudomonas* genus (Tayeb *et al.*, 2005; Passo, 2009).

The results of the present study indicated that, although *X. campestris* strains were associated to the disease observed in Mauritius in 2009 and 2012, pseudomonads might also contribute to the disease phenotype observed in the field. The simultaneous occurrence of *X. campestris* and *P. syringae* pv. *maculicola* strains causing leaf spot symptoms in field-grown brassicas has been previously reported (Zhao *et al.*, 2000a; Zhao *et al.*, 2000b). It would be interesting to test whether co-inoculation of plants with the *X. campestris* and *Pseudomonas* strains described in this study would result in a more severe leaf spot and blight disease phenotype as described from observations in the field. This would help to confirm whether the atypical symptoms observed in the field are a result of a synergistic interaction

between different pathogens as described for some plant diseases (Lamichhane & Venturi, 2015).

In the present study, it was confirmed that the pathotype strain of *Xcr* NCPPB1946 (added to the HRI collection under the accession HRI8803) is an authentic *Xcr* strain because it caused typical leaf spot symptoms in several hosts known to be susceptible to *Xcr*. These observations are in agreement with a few studies that reported leaf spot symptoms caused by clones of the pathotype strain of *Xcr* obtained from different collections including the National Collection of Plant Pathogenic Bacteria (NCPPB) (Tamura *et al.*, 1994; Benoit *et al.*, 2000; Fargier & Manceau, 2007; Loreti *et al.*, 2011). Furthermore, the results of the present study confirmed that the strain HRI3883, which has been described as a clone of the pathotype strain of *Xcr* (NCPPB1946) and re-identified as *Xcc* (Vicente *et al.*, 2001), has been mislabelled and it is not a true clone of the *Xcr* pathotype strain.

So far, three *Xcr* races have been described based on interactions with *Brassicaceae* plant differentials (Vicente *et al.*, 2006). The *Xcr* strains that were race-typed in this study showed interaction patterns with the *Brassicaceae* differentials similar to those previously described. The newly received pathotype strain of *Xcr* NCPPB1946 (HRI8803) was identified as race 1 contrarily to the results presented by Fargier & Manceau (2007) that identified this strain as race 3. Among the remaining *Xcr* strains studied, the strains from Italy (Loreti *et al.*, 2011) were identified as race 3 and the strain from Africa was identified as race 2. The strain from Africa (HRI7803) has been previously identified as *Xcc* (Vicente *et al.*, 2001) but the fact that it grouped with other *Xcr* strains in a *gyrB* phylogenetic tree (J. Mulema, pers. comm.) suggested that this strain might have been misidentified. In the present study, this strain caused typical symptoms of leaf spot disease caused by *Xcr* (leaf spots and dark sunken lesions in petioles) and, therefore, it was identified as *Xcr*. This result suggests the presence of *Xcr* in Africa where it has not been previously reported. The strain from the UK was identified as *Xcr* race 2 although this strain was different from the race-type of *Xcr* race 2 in the interaction with the oilseed rape COB60 as it only produced very few spots in this plant. It is important to highlight here that the identification of this *Xcr* strain constitutes the first evidence of the presence of this pathogen in the UK (D. Stead and J. Vicente, pers. comm.).

Finally, the pathogenicity tests with strain HRI8804 received as *Xcr* from the NCPPB collection (strain NCPPB3566; isolated from radish in India), indicated that this strain should be identified as *Xcc* race 1 because it caused vascular infections typical of *Xcc* and produced interactions with plant differentials identical to those of race 1 (Vicente & Holub, 2013). As no publications were found describing this strain, it is possible that its identification as *Xcr* was not confirmed since 1988 when it was first deposited in the NCPPB collection.

# CHAPTER 5. Exploring whole-genome sequencing data of *Xanthomonas campestris* to identify candidate determinants of pathogenic variation in *Brassicaceae* hosts

## 5.1 INTRODUCTION

*Xanthomonas campestris* pv. *raphani* (*Xcr*) and *campestris* (*Xcc*) are genetically close pathogens (Fargier *et al.*, 2011) that infect common hosts within the *Brassicaceae* family including *Brassica* spp. and *Arabidopsis thaliana* but cause distinct diseases, bacterial leaf spot and black rot respectively. *Xcr* strains typically enter the host via stomata, colonize the parenchyma tissue, but not the vascular system, and cause leaf spots (White, 1930; Tamura *et al.*, 1994; Vicente *et al.*, 2006); whereas *Xcc* strains typically enter the host via hydathodes, colonize the xylem elements of the vascular system, spread systemically within the host and cause blackening of veins and extended chlorotic/necrotic lesions in leaves (Williams, 1980; Bretschneider *et al.*, 1989; Alvarez, 2000). *Xcc* strains may also cause small leaf spots, but these lesions are distinct from those caused by *Xcr* strains, and symptoms progress to extended chlorotic/necrotic lesions as observed in inoculations with strains isolated in Mauritius and South Carolina (Chapter 4) and other *Xcc* strains (Vicente *et al.*, 2006).

Our understanding of the molecular determinants of the distinct modes of pathogenesis of these pathovars is at an early stage. Pathogenicity determinants have been identified in *Xcc* (Alvarez, 2000; Ryan *et al.*, 2011; Vicente & Holub, 2013) but to date, little is known regarding the pathogenicity determinants of *Xcr*. Comparative analyses of genome sequences of strains of each pathovar have begun to provide insights into candidate determinants of their distinct mode of pathogenesis. Bogdanove *et al.* (2011) published the first complete genome sequence of a *Xcr* strain (756C) and reported differences between this strain and three *Xcc* strains (ATCC33913, B100 and LMG8004), as for example in the repertoire of genes predicted or known to encode type III effectors (T3Es), cyclic di-GMP signalling proteins and adhesins. More recently, a draft genome sequence of a second *Xcr* strain was published and differences in the T3E gene repertoire were reported between two *Xcr* strains and eight *Xcc* strains (Roux *et al.*, 2015).

In addition, races have been described within each pathovar based on compatible and incompatible interactions with plant differential lines of five *Brassica* species (*B. oleracea*, *B. carinata*, *B. rapa*, *B. juncea* and *B. napus*), and *Raphanus sativus* (radish) for *Xcr* races (Vicente *et al.*, 2001; Vicente *et al.*, 2006;

Fargier & Manceau, 2007; Vicente & Holub, 2013). Gene-for-gene models have been postulated to explain these interactions with predictions for a minimum number of matching avirulence/resistant gene pairs (see further description in Table 2, Chapter 1). For *Xcr*, three races are currently described and at least two avirulence genes (*A1r* and *A2r*) are predicted within the pathovar (Vicente *et al.*, 2006); for *Xcc*, nine races have been described; hence the current gene-for-gene model is more complex and at least five avirulence genes (*A1c* to *A5c*) are predicted within this pathovar (Vicente & Holub, 2013) (Table 27).

**Table 27**. Summary of hypothesised presence/absence patterns of avirulence genes across races of *Xanthomonas campestris* pathovars *raphani* and *campestris*, derived from the published gene-for-gene models to explain the interactions between races of each pathovar and *Brassicaceae* differentials [a]

| **Avirulence genes** | **Races** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ***Xcr*** | | | ***Xcc*** | | | | | | | | |
| | **1** | **2** | **3** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** |
| ***A1r*** | + | − | − | | | | | | | | | |
| ***A2r*** | − | + | − | | | | | | | | | |
| ***A1c*** | | | | + | − | + | + | − | − | − | + | + |
| ***A2c*** | | | | − | + | − | − | − | − | − | + | − |
| ***A3c*** | | | | − | + | + | − | + | − | − | + | + |
| ***A4c*** | | | | − | − | − | + | − | − | − | − | + |
| ***A5c*** | | | | − | − | − | − | − | − | + | − | − |
| No. of strains [b] | 3 | 3 | 3 | 4 | 1 | 2 | 6 | 2 | 5 | 1 | 1 | 1 |

+, gene present; −, gene absent (or highly divergent in sequence).

[a] Adapted from Vicente *et al.* (2006) and Vicente & Holub (2013). See further description in Table 2, Chapter 1.

[b] Number of strains of each race included in the present study.

The molecular basis for the race structure of *Xcr* and *Xcc* remains poorly characterized. No avirulence genes have thus far been described to explain the race variation within *Xcr*. In *Xcc*, a few genes have been reported to have an avirulence function including two (*xopAH* and *xopE*) that may correspond to avirulence genes predicted in the gene-for-gene model of this pathovar (Vicente & Holub, 2013; Table 2, Chapter 1). The gene *xopAH* (syn. *avrXccFM*, *avrXccC*) of a race 3 strain (ATCC33913, syn. 528) has been shown to confer avirulence in the differential host *B. juncea* cv. Florida Broad Leaf Mustard (FBLM) (Castaneda *et al.*, 2005). Similarly, He *et al.* (2007) showed that *xopAH* of a race 9 strain (LMG8004) confers

avirulence in a cultivar of the same species (*B. juncea* cv. Guangtou). This gene may therefore correspond to the avirulence gene *A1c* predicted in *Xcc* races 1, 3, 4, 8 and 9 (Table 27), which are all avirulent in *B. juncea* cv. FBLM (Vicente *et al*., 2013; Table 2, Chapter 1). The gene *xopE* (syn. *avrXccE1*) of strain LMG8004 (race 9) has been shown to confer avirulence on Chinese cabbage (*B. rapa* subsp. *pekinensis*) (He *et al.*, 2007) and may correspond to the *A4c* gene which is predicted to confer avirulence to *Xcc* races 4 and 9 (Table 27) in a host differential genotype of the same species (*B. rapa* cv. Just Right Hybrid Turnip) (Vicente *et al*., 2013; Table 2, Chapter 1).

At the beginning of this study, complete reference genome sequences of one *Xcr* strain (Bogdanove *et al.*, 2011) and three complete genomes of *Xcc* strains of race 1 (B100) (Vorhölter *et al.*, 2008), race 3 (ATCC33913) (da Silva *et al.*, 2002) and race 9 (LMG8004) (Qian *et al.*, 2005) were publically available. During this study, additional *X. campestris* genome sequences became available including draft genomes of one *Xcc* race 1 (Xca5) (Bolot *et al.*, 2013a) and three *Xcc* strains of unknown race (CN14, CN15, CN16) (Bolot *et al.*, 2013b).

The present study aimed to explore whole-genome sequencing data of multiple strains of *Xcr* and *Xcc* including representatives of all races defined within both pathovars, to identify candidate determinants of the distinct modes of pathogenesis of *Xcr* and *Xcc* as well as avirulence of races in *Brassicaceae* plant differentials. In this study, it was hypothesized that genes differentially present/absent (or highly divergent) between strains of these pathovars may include key determinants of their distinct mode of pathogenesis. Similarly, genes differentially present/absent (or highly divergent) across races as predicted in Table 27 from the published gene-for-gene models, may include determinants of avirulence. For this purpose, whole-genome sequencing data was obtained for a set of 32 *X. campestris* strains (including eight *Xcr*, 20 *Xcc* and four other *X. campestris* strains) and used together with the publicly available reference complete genomes of one *Xcr* and three *Xcc* strains. This collection of strains included examples isolated from different hosts, in different continents and years, with representatives of all races defined within *Xcr* and *Xcc* either race-typed in previous studies or in this study (Chapter 4).

The specific objectives of this study were to:

a) Determine the phylogenetic relatedness of the *X. campestris* strains used in this study.

b) Identify genes that are differentially present/absent (or highly divergent in sequence) between strains of *Xcr* and *Xcc*.

c) Identify genes that are differentially present/absent (or highly divergent) in races of *Xcr* or *Xcc* according to the predictions for avirulence determinants based on published gene-for-gene models (as presented in Table 27).

## 5.2 MATERIALS AND METHODS

### 5.2.1 Bacterial strains

Whole-genome sequencing data from 36 strains of *X. campestris* was used in this study. This included data from 32 strains (24 strains sequenced in this study and eight strains for which unpublished data was provided by M. Grant and D. Studholme, University of Exeter, UK), and four strains for which there was a publicly available complete genome sequence (Table 28).

The selected strains included nine *Xcr* and 23 *Xcc* strains, all of which have been race-typed in previous studies or in this study (Chapter 4), and included representatives of all races described within *Xcr* and *Xcc*. The three known races of *Xcr* were represented by three strains each, including the pathotype strain (HRI8803) race-typed as described in Chapter 4. The most common races of *Xcc* (races 1 and 4) and the broadly virulent *Xcc* race 6 were represented by at least four strains each, including three blight causing strains isolated from disease outbreaks in Mauritius (strains HRI8506 and 8821, race 4) and South Carolina (strain HRI8806, race 6) which were race-typed in the present study (Chapter 4).

The set of strains also included four additional *X. campestris* strains that have been isolated from *Brassicaceae* hosts, but have distinct pathogenic features from *Xcr* and *Xcc* strains. This group included three strains that cause vascular diseases in ornamental crucifers and have been reported to be non-pathogenic on *Brassica* spp. (one strain that causes bacterial blight in garden stock and is identified as *X. campestris* pv. *incanae* (HRI6378) (Vicente *et al.*, 2001; Fargier & Manceau, 2007), one strain from wallflower reported to be pathogenic only on this host (HRI5219) (Vicente *et al.*, 2001) and one strain from candytuft pathogenic on this host and radish (HRI6375) (Vicente *et al.*, 2001; Fargier & Manceau, 2007)), as well as one strain from horseradish (HRI6376). This last strain was received as *X. campestris* pv. *armoraciae* (National Collection of Plant Pathogenic Bacteria; http://ncppb.fera.defra.gov.uk/), but it has been reported to be non-pathogenic on horseradish (contrarily to the definition of this pathovar) and other *Brassicaceae* plants tested (Vicente *et al.*, 2001; Vicente *et al.*, 2006; Fargier & Manceau, 2007).

**Table 28**. List of *Xanthomonas campestris* strains used in genome-wide comparisons

| Strain accession (other designation) | Isolation | | | Source (reference[a]) | Genome sequencing data | |
|---|---|---|---|---|---|---|
| | Host | Geographic origin | Year | | Status | Source [RefSeq accession] |
| ***Xanthomonas campestris* pv. *raphani*** | | | | | | |
| **Race 1** | | | | | | |
| HRI6490[Rt] | *Brassica oleracea* var. *botrytis* (cauliflower) | France | 1995 | HRI (Vicente *et al.*, 2006) | Contig | This study |
| HRI8298 | *Raphanus sativus* (radish) | Minnesota, USA | na | HRI (Vicente *et al.*, 2006) | Contig | This study |
| HRI8803[Pt] (NCPPB1946) | *R. sativus* (radish) | USA | 1940 | NCPPB (this study) | Contig | This study |
| **Race 2** | | | | | | |
| HRI6520 | *B. rapa* var. *pekinensis* (Chinese cabbage) | Nagano, Japan | 1986 | HRI (Vicente *et al.*, 2006) | Contig | This study |
| HRI8305[Rt] | *B. rapa* var. *perviridis* (spinach mustard) | Oklahoma, USA | 1995 | HRI (Vicente *et al.*, 2006) | Contig | E. Holub and J. Vicente (unpublished) |
| HRI8474 (P764, NCPPB4451) | *Erysimum cheiri* (wallflower) | UK | na | NCPPB, D. Stead and J. Vicente (this study) | Contig | This study |
| **Race 3** | | | | | | |
| HRI6519[Rt] | *R. sativus* (radish) | Shizuoka, Japan | 1985 | HRI (Vicente *et al.*, 2006) | Contig | This study |
| HRI8299 | *Solanum lycopersicum* (tomato) | Ontario, Canada | na | HRI (Vicente *et al.*, 2006) | Contig | This study |
| 756C (HRI8503) | *B. oleracea* var. *capitata* | East Asia | na | na (Vicente *et al.*, 2006; Fargier & Manceau, 2007) | Complete genome | Bogdanove *et al.* (2011) [NC_017271.1] |
| ***X. campestris* pv. *campestris*** | | | | | | |
| **Race 1** | | | | | | |
| HRI3811[Rt] | *B. oleracea* | USA | na | HRI (Vicente *et al.*, 2001) | Contig | E. Holub and J. Vicente (unpublished) |
| HRI6195 | *B. oleracea* var. *acephala* (kale) | Lourinhã, Portugal | 1997 | HRI (Vicente *et al.*, 2001) | Contig | This study |
| HRI8333 | *B. oleracea* var. *capitata* (cabbage) | Spain | 2003 | HRI (J. Vicente, pers. comm.) | Contig | This study |
| B100 | *B. oleracea* | Italy | na | na (J. Vicente, pers. comm.) | Complete genome | Vorhölter *et al.* (2008) [NC_010688.1] |
| **Race 2** | | | | | | |
| HRI3849A[Rt] | *B. oleracea* var. *botrytis* (cauliflower) | USA | na | HRI (Vicente *et al.*, 2001) | Contig | D. Studholme and M. Grant (unpublished) |
| **Race 3** | | | | | | |
| HRI8819 | *B. oleracea* (wild plant) | Isle of Purbeck, UK | 2012 | E. Holub (J. Vicente, pers. comm.) | Contig | This study |
| ATCC33913[Pt, Rt] | *B. oleracea* var. *gemmifera* (Brussels sprouts) | UK | 1957 | na (Vicente *et al.*, 2001) | Complete genome | da Silva *et al.* (2002) [NC_003902.1] |
| **Race 4** | | | | | | |
| HRI1279A[Rt] | *B. oleracea* var. *capitata* (cabbage) | Cornwall, UK | 1984 | HRI (Vicente *et al.*, 2001) | Contig | D. Studholme and M. Grant (unpublished) |

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| Strain accession (other designation) | Isolation | | | Source (reference[a]) | Genome sequencing data | |
|---|---|---|---|---|---|---|
| | Host | Geographic origin | Year | | Status | Source [RefSeq accession] |
| HRI6312A | *B. oleracea* var. *botrytis* (cauliflower) | California, UK | na | HRI (Vicente *et al.*, 2001) | Contig | This study |
| HRI7758 | *B. oleracea* var. *tronchuda* (cabbage) | Minas Gerais, Brazil | 1999 | HRI (Vicente *et al.*, 2001) | Contig | This study |
| HRI7806 | *B. oleracea* var. *capitata* (cabbage) | Northam, South Africa | 1998 | HRI (Vicente *et al.*, 2001) | Contig | This study |
| HRI8506 | *B. oleracea* var. *capitata* (cabbage) | Mauritius | 2009 | R. Lobin (this study) | Contig | This study |
| HRI8821 | *B. oleracea* var. *botrytis* (cauliflower) | Mauritius | 2012 | This study (this study) | Contig | This study |
| **Race 5** | | | | | | |
| HRI3880$^{Rt}$ (NCPPB2986, CFBP6865) | *B. oleracea* var. *capitata* (cabbage) | Australia | 1975 | HRI (Vicente *et al.*, 2001) | Contig | D. Studholme and M. Grant (unpublished) |
| HRI6382 | *B. rapa* | Canada | 1953 | HRI (Vicente *et al.*, 2001) | Contig | This study |
| **Race 6** | | | | | | |
| HRI6181$^{Rt}$ | *B. rapa* | Sardoal, Portugal | 1996 | HRI (Vicente *et al.*, 2001) | Contig | This study |
| HRI6185 | *B. rapa* | Castelo Branco, Portugal | 1996 | HRI (Vicente *et al.*, 2001) | Contig | This study |
| HRI8450B | *B. oleracea* var. *capitata* (cabbage) | Dhankuta, Nepal | 2001 | HRI (Jensen *et al.*, 2010) | Contig | This study |
| HRI8497 | *B. rapa* (accession of wild plant) | UK | 2008 | HRI (J. Vicente, pers. comm.) | Contig | This study |
| HRI8806 (418) | *B. juncea* cv. Green Wave (mustard) | Pelion, South Carolina, USA | 2005 | P. Wechter (Wechter *et al.* 2008; this study) | Contig | This study |
| **Race 7** | | | | | | |
| HRI8450A (N47) | *B. oleracea* var. *capitata* (cabbage) | Dhankuta, Nepal | 2001 | HRI (Jensen *et al.*, 2010) | Contig | This study |
| **Race 8** | | | | | | |
| HRI8815$^{Rt}$ (CFBP1124) | *B. oleracea* var. *botrytis* (cauliflower) | France | 1967 | CFBP (Fargier & Manceau, 2007) | Contig | This study |
| **Race 9** | | | | | | |
| LMG8004 | *B. oleracea* var. *botrytis* (cauliflower) | Sussex, UK | 1958 | na (Fargier & Manceau, 2007) | Complete genome | Qian *et al.* (2005) [NC_007086.1] |
| **Other *X. campestris*** | | | | | | |
| *X. campestris* pv. *incanae* (vascular pathogen of garden stock) | | | | | | |
| HRI6378 (NCPPB1934, CFBP1438) | *Matthiola incana* (garden stock) | USA | 1949 | HRI (Vicente *et al.*, 2001; Fargier & Manceau, 2007) | Contig | D. Studholme and M. Grant (unpublished) |
| *X. campestris* (vascular pathogen of wallflower) | | | | | | |
| HRI5219A (NCPPB2517) | *Erysimum cheiri* (wallflower) | UK | 1970 | HRI (Vicente *et al.*, 2001) | Contig | D. Studholme and M. Grant (unpublished) |

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| Strain accession (other designation) | Isolation | | | Source (reference[a]) | Genome sequencing data | |
|---|---|---|---|---|---|---|
| | Host | Geographic origin | Year | | Status | Source [RefSeq accession] |
| *X. campestris* (vascular pathogen of candytuft) | | | | | | |
| HRI6375 (NCPPB347, CFBP3838) | *Iberis* sp. (candytuft) | Tanzania | 1954 | HRI (Vicente *et al.*, 2001; Vicente *et al.*, 2006; Fargier & Manceau, 2007) | Contig | D. Studholme and M. Grant (unpublished) |
| *X. campestris* pv. *armoraciae* (reported to be non-pathogenic) | | | | | | |
| HRI6376 [b] (NCPPB1930, CFBP5824) | *Armoracia rusticana* (horseradish) | New Zealand | 1939 | HRI (Vicente *et al.*, 2001; Vicente *et al.*, 2006; Fargier & Manceau, 2007) | Contig | This study |

Abbreviations: CFBP, French Collection of Plant associated Bacteria, Angers, France; HRI, formerly Horticulture Research International, now University of Warwick Crop Centre, Wellesbourne, UK; LMG, Laboratory for Microbiology, Faculty of Sciences, Ghent University, Ghent, Belgium; na, not available; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, UK; Pt, pathotype; Rt, race-type.

[a] Reference for race typing and/or pathogenic characterization.

[b] Strain identified as *X. campestris* pv. *armoraciae* (National Collection of Plant Pathogenic Bacteria; http://ncppb.fera.defra.gov.uk/) but it has been reported to be non-pathogenic on horseradish and other *Brassicaceae* plants tested (Vicente *et al.*, 2001; Vicente *et al.*, 2006; Fargier & Manceau, 2007).

Bacterial cultures were isolated in this study or were obtained from: the bacterial collection at the University of Warwick Crop Centre (formerly Horticulture Research International, HRI; Wellesbourne, UK), National Collection of Plant Pathogenic Bacteria (NCPPB; Fera, York, UK), French Collection of Plant associated Bacteria (CFBP; Angers, France), R. Lobin (Agricultural Research and Extension Unit, Plant Pathology Division, Mauritius) and P. Wechter (USDA-ARS, Charleston, South Carolina, USA), as specified in Table 28.

### 5.2.2 DNA extraction

DNA was extracted from 24 bacterial strains (listed in Table 28 as sequenced in this study). DNA extractions from 14 strains were performed by J. Vicente (University of Warwick, UK). Bacterial strains were grown on King's B medium (King *et al.*, 1954) for 24 to 48 h at 28 °C. Bacterial suspensions were prepared in sterile ultrapure water and density adjusted visually to 3 to 4 McFarland turbidity standards (*ca.* $10^8$ cfu/ml) (Király *et al.*, 1974). Total bacterial DNA was isolated using the

DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's instructions for purification of DNA from Gram-negative bacteria. The concentration and quality of the extracted DNA were checked using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

### 5.2.3 Genome sequencing

The genomic DNA of 24 strains was sent for short-read sequencing to the Genomics Facility of the School of Life Sciences of the University of Warwick (UK) or to the Sequencing Service of the University of Exeter (UK). Paired-end short read sequencing was performed using an Illumina GAIIx analyser for genomes sequenced at Warwick and an Illumina MiSeq analyser for genomes sequenced at Exeter. Paired-end reads of 70 bp or 251 bp were obtained from DNA samples sequenced at Warwick or Exeter, respectively.

The quality of genomic sequence reads was assessed using fastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adapters and poor quality reads were trimmed and filtered using fastq-mcf (Aronesty, 2011). The number of reads, sequencing type, average read length and depth of coverage of short-read sequencing data used in this study, are summarized in Appendix 13. The depth of coverage was estimated for each sample by multiplying the number of sequence reads by the average read length, and dividing this number by the estimated size of the *Xanthomonas campestris* genome (5 Mb; da Silva *et al.*, 2002; Qian *et al.*, 2005; Vorhölter *et al.*, 2008; Bogdanove *et al.*, 2011), and varied from 20 to 671-fold. This gives an estimation of the number of times that each nucleotide was sequenced under the assumption that sequence reads are randomly distributed across the genome (Sims *et al*., 2014).

### 5.2.4 Genome assemblies and annotation

*De novo* genome assemblies were generated for 28 strains for which paired-end sequence reads were obtained (strains listed in Appendix 13). These assemblies were generated by the Exeter Sequencing Service or by J. Harrison and D. Studholme (University of Exeter, UK), using Velvet (Zerbino & Birney, 2008). For

each assembly, the shortest, median and longest contig sizes, total sequence size and GC content were determined using Geneious software (Kearse *et al.*, 2012), and the N50 (*i.e.* the length for which the collection of all contigs of that length or longer contains at least half of the sum of the lengths of all contigs) was calculated using a Perl script developed by L. Baxter (University of Warwick, UK). These statistics are summarized in Appendix 14.

The *de novo* genome assemblies were annotated using the RAST online annotation pipeline version 4.0 (Aziz *et al.*, 2008; http://rast.nmpdr.org/). This involved gene prediction and assignment of biological functions.

Reference-guided assemblies were generated for all 32 newly sequenced strains (listed in Appendix 13) by mapping sequencing reads to a reference published complete genome using the 'Map to reference' tool in Geneious software with default parameters. The complete genome of *Xcr* strain 756C (RefSeq accession NC_017271.1) (Bogdanove *et al.*, 2011) was used as a reference to generate genome assemblies for all *Xcr* strains; and the complete genome of *Xcc* strain ATCC33913 (RefSeq accession NC_003902.1) (da Silva *et al.*, 2002) was used as a reference to generate genome assemblies for the remaining strains (*Xcc* and *X. campestris* strains).

### 5.2.5 Phylogenetic analysis

Phylogenetic trees were generated based on sequences of two different sets of genes listed in Table 29. These genes were chosen because they have been used as phylogenetic markers in previous studies. This included a set of four genes (three housekeeping genes, *dnaK*, *rpoD* and *gyrB*; and one structural gene, *fyuA*) that have been used for phylogenetic inference of *X. campestris* pathovars (Fargier *et al.*, 2011) and *Xanthomonas* species (Young *et al.*, 2008), and a set of 31 housekeeping genes that have been used for phylogenetic inference of bacterial and archaeal genomes (Wu *et al.*, 2009).

Complete nucleotide sequences of each gene were retrieved from genome assemblies of 36 strains under study (listed in Table 28) using Geneious software. In addition, gene sequences were also retrieved from four published draft genomes of *X.*

*campestris* pv. *campestris* (strains CN14, 15 and 16 (Bolot *et al.*, 2013b) and strain Xca5 (Bolot *et al.*, 2013a)) as well as three published genomes of *X. oryzae* pv. *oryzae* (strain KACC10331) (Lee *et al.*, 2005), *X. euvesicatoria* (strain 85-10) (Thieme *et al.*, 2005), and *X. axonopodis* pv. *citri* (strain 306) (da Silva *et al.*, 2002). These three latter strains were selected as an outgroup because their phylogenetic positions within the *Xanthomonas* genus are distant from the *X. campestris sensu stricto* group (Young *et al.*, 2008).

Sequences of each gene were aligned using the MUSCLE algorithm (Edgar, 2004) implemented in Geneious software. Sequence alignments of each gene were then concatenated using the same software. Phylogenetic analyses of multi-gene sequence datasets were performed using MEGA6 software (Tamura *et al.*, 2013). The model that best described the nucleotide substitution pattern of each dataset was selected in this software. Phylogenetic trees were constructed by using the Maximum Likelihood method (Felsenstein, 1981) and based on the best-fit substitution model. The reliability of each node in the trees was estimated with 1000 bootstrap replicates (Felsenstein, 1985).

**Table 29**. List of genes used in phylogenetic analyses

| Gene name | Protein name | Length (bp) | Reference locus [a] |
|---|---|---|---|
| **Set of four genes previously used by Fargier *et al.* (2011) and Young *et al.* (2008)** | | | |
| *dnaK* | Chaperone protein DnaK | 1929 | XCC1474 |
| *fyuA* | TonB-dependent receptor | 2475 | XCC3358 |
| *gyrB* | DNA gyrase subunit B | 2445 | XCC0004 |
| *rpoD* | RNA polymerase sigma-70 factor | 1875 | XCC3736 |
| **Set of 31 genes previously used by Wu *et al.* (2009)** | | | |
| *dnaG* | DNA primase | 1749 | XCC3820 |
| *frr* | Ribosome recycling factor | 558 | XCC1370 |
| *infC* | Initiation factor IF-3 | 480 | XCC2462 |
| *nusA* | N utilization substance protein A | 1512 | XCC2512 |
| *pgk* | Phosphoglycerate kinase | 1176 | XCC3188 |
| *pyrG* | CTP synthetase | 1665 | XCC1697 |
| *rplA* | 50S ribosomal protein L1 | 699 | XCC0885 |
| *rplB* | 50S ribosomal protein L2 | 828 | XCC0898 |
| *rplC* | 50S ribosomal protein L3 | 651 | XCC0895 |
| *rplD* | 50S ribosomal protein L4 | 606 | XCC0896 |
| *rplE* | 50S ribosomal protein L5 | 543 | XCC0907 |
| *rplF* | 50S ribosomal protein L6 | 528 | XCC0910 |
| *rplK* | 50S ribosomal protein L11 | 429 | XCC0884 |
| *rplL* | 50S ribosomal protein L7/L12 | 366 | XCC0887 |
| *rplM* | 50S ribosomal protein L13 | 429 | XCC0476 |
| *rplN* | 50S ribosomal protein L14 | 369 | XCC0905 |
| *rplP* | 50S ribosomal protein L16 | 414 | XCC0902 |
| *rplS* | 50S ribosomal protein L19 | 408 | XCC1202 |
| *rplT* | 50S ribosomal protein L20 | 360 | XCC2460 |
| *rpmA* | 50S ribosomal protein L27 | 261 | XCC1150 |
| *rpoB* | RNA polymerase beta subunit | 4164 | XCC0888 |
| *rpsB* | 30S ribosomal protein S2 | 828 | XCC1375 |
| *rpsC* | 30S ribosomal protein S3 | 735 | XCC0901 |
| *rpsE* | 30S ribosomal protein S5 | 543 | XCC0912 |
| *rpsI* | 30S ribosomal protein S9 | 393 | XCC0477 |
| *rpsJ* | 30S ribosomal protein S10 | 312 | XCC0894 |
| *rpsK* | 30S ribosomal protein S11 | 393 | XCC0917 |
| *rpsM* | 30S ribosomal protein S13 | 357 | XCC0916 |
| *rpsS* | 30S ribosomal protein S19 | 270 | XCC0899 |
| smpB | Small protein B | 504 | XCC1466 |
| *tsf* | Elongation factor Ts | 879 | XCC1374 |

[a] Locus in the complete genome sequence of *Xanthomonas campestris* pv. *campestris* strain ATCC33913 (RefSeq accession NC_003902.1; da Silva *et al.*, 2002).

### 5.2.6 Genome-wide identification of candidate determinants of distinct pathogenic features of *Xanthomonas campestris* pathovars *raphani* and *campestris*, and avirulence of races within these pathovars

Genome-wide searches were performed for: 1) genes that are present in all *Xcr* strains, but absent (or highly divergent) in all *Xcc* strains; 2) genes that are present in all *Xcc* strains, but absent (or highly divergent) in all *Xcr* strains; and 3) genes that correspond with presence/absence patterns of avirulence determinants predicted in *Xcr* and *Xcc* races as shown in Table 27 (Section 5.1). In this last case, searches were only performed if there were at least three strains in each comparative group, which was possible for the two predicted *Xcr* avirulence genes (*A1r* and *A2r*), and three of five predicted *Xcc* avirulence genes (*A1c*, *A3c* and *A4c*, but not for *A2c* and *A5c*) (Table 27, Section 5.1).

To perform these searches, genomic sequence reads from each strain were aligned against a reference pan-genome rather than comparing genome assemblies, following a method described by Aritua *et al.* (2015). This approach was taken to allow detection of genes that could be missing in the genome assemblies of some strains. The reference pan-genome was built based on predicted genes from 32 *X. campestris* genome assemblies. These included *de novo* genome assemblies obtained for 28 newly sequenced strains (listed in Appendix 14) and published complete genomes of one *Xcr* strain (756C) and three *Xcc* strains (ATCC33913, B100 and LMG8004) (Table 28). Sequences of all predicted genes were grouped at 95% sequence identity using UCLUST (Edgar, 2010) and a total of 11159 clusters resulted from this analysis.

The consensus sequences generated from a multiple alignment of the constituent sequences of each cluster were used to create the pan-genome. For simplicity, I will refer to these as pan-genes, each being a representative of highly similar gene sequences. The genomic sequence reads obtained for all 32 newly sequenced strains under study and simulated sequence reads[2] generated from the four published complete genomes mentioned above, were then aligned against the reference pan-genome using BWA-MEM (Li & Durbin, 2009; Li, 2014). The percentage of the length of each pan-gene that was covered by aligned genomic

[2] Simulated sequence reads generated by randomly sampling the published complete genome assemblies using a costum Perl script developed by J. Harrison (University of Exeter, UK).

sequence reads (breadth of coverage, BC) was determined using coverageBed from the BEDtools package (Quinlan & Hall, 2010). This analysis was carried out by J. Harrison and supervised by D. Studholme (University of Exeter, UK).

The BC values for the 11159 pan-genes by genomic sequence reads of each of the 36 genomes under study (Table 28) were combined in a Microsoft Excel worksheet. This worksheet was then used to search for group-associated pan-genes (*i.e.* those that showed BC >80% for all strains within one defined group and BC <20% for all strains within a defined comparative group). These thresholds were set assuming that high coverage of a pan-gene's length by genomic sequence reads suggests presence of each gene in a strain; and that low coverage suggests its absence (or high divergence in sequence). Pan-genes of length less than 300 bp were excluded at this stage from further analysis.

To refine the list of pan-genes that could distinguish between groups of strains, sequence similarity searches using BLASTN (with default parameters in Geneious software) were performed with each pan-gene sequence against the genome assemblies of strains in the comparative group. Pan-genes that showed hits with query coverage ≥25% at any level of sequence identity were excluded. This second step was performed to further exclude pan-genes at this stage that share intermediate sequence identity with genes in the genome assemblies of members of the comparative group, which had not been detected in the alignments with sequence reads.

To infer which gene is represented by each selected pan-gene, one of the longest gene sequences was selected from the cluster from which the pan-gene was derived (as a reference sequence for each gene) and the gene product predicted in the annotation of that sequence was used. Sequences sharing the highest identity over the full length of each selected sequence were extracted from genome assemblies of strains within each group under study, to confirm the presence of each gene. This was performed using the tool 'annotate and predict' in Geneious software. For the newly sequenced strains, sequences were extracted either from *de novo* assemblies or reference-guided assemblies (when sequences were not found in *de novo* assemblies or when *de novo* assemblies were not available for a particular strain). Nucleotide sequences were translated into amino acids sequences to reveal stop codons. When premature stop codons were identified in a particular sequence, the coding sequence

was confirmed using the respective annotated genome assembly. Multiple alignments of amino acid sequences were generated for sequence comparison using Geneious software.

### 5.2.7 Sequence similarity searches

Similarity searches of selected gene reference sequences were performed against the RefSeq protein database (NCBI website; http://www.ncbi.nlm.nih.gov/) and a custom database of all predicted/known T3E protein sequences from whole genome sequenced *Xanthomonas* spp. strains listed in the *Xanthomonas* Resource database (http://www.xanthomonas.org/t3e.html, accessed in October 2015; White *et al.*, 2009). Similarity searches were performed using BLASTX (Altschul *et al.*, 1997). Searches against the RefSeq protein database were performed in the NCBI website and searches against the custom T3E database were performed using Geneious software.

## 5.3 RESULTS

### 5.3.1 Phylogenetic analysis

To determine the genetic relatedness among the *X. campestris* strains under study, phylogenetic trees were generated based on concatenated sequences of two different sets of genes (four and 31 genes) that have been used as phylogenetic markers in previous studies (Figures 30 and 31). Two main clades were conserved in both trees and each had strong bootstrap support (clades A and B indicated in Figures 30 and 31). All *Xcc* strains grouped together with one vascular pathogen of candytuft (strain HRI6375) in clade A. The blight causing strains from Mauritius (HRI8506 and 8821) and South Carolina (HRI8806) (Wechter *et al.*, 2008) characterized in Chapter 4, were also included in this clade further supporting their identification as *Xcc*. The two vascular pathogens of ornamental crucifers (*X. campestris* pv. *incanae* strain, HRI6378; and a strain from wallflower, HRI5219A) and the *X. campestris* pv. *armoraciae* strain grouped together in clade B.

All *Xcr* strains fell outside of clades A and B in both trees, but their phylogenetic relationships were not clearly resolved. In the phylogenetic tree based on four genes (Figure 30), all *Xcr* strains grouped in one single clade but this clade had low bootstrap support (45%; clade C). In the phylogenetic tree based on 31 genes (Figure 31), most strains grouped in two separate clades (clades C and D) of which only one had high bootstrap support (88%, clade C), and one strain separated from all the other *X. campestris* strains in that tree. Only one clade supported by high bootstrap value was found consistent in both trees and comprised the *Xcr* strains 756C, HRI6520 and HRI8305. The branching order between the clades A and B and clades comprising the *Xcr* strains also varied between trees and the bootstrap was low in some branches.

Races of *Xcr* and *Xcc* did not group consistently together, although some *Xcc* strains of the same race grouped together with high bootstrap in both trees (clades highlighted in red in Figures 30 and 31).

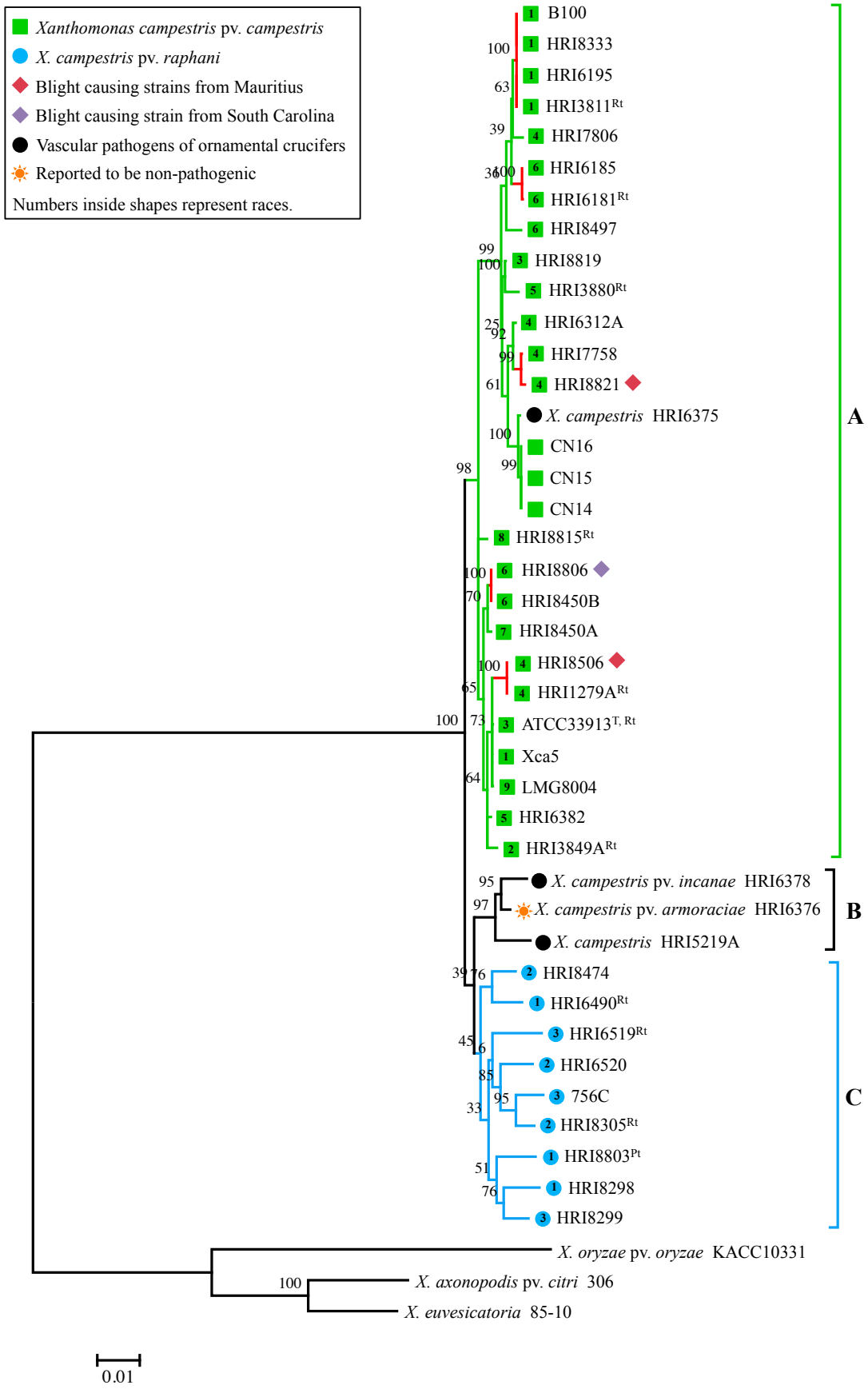
Xanthomonas campestris pv. campestris
X. campestris pv. raphani
Blight causing strains from Mauritius
Blight causing strain from South Carolina
Vascular pathogens of ornamental crucifers
Reported to be non-pathogenic
Numbers inside shapes represent races.
B100
HRI8333
HRI6195
HRI3811Rt
HRI7806
HRI6185
HRI6181Rt
HRI8497
HRI8819
HRI3880Rt
HRI6312A
HRI7758
HRI8821
X. campestris HRI6375
CN16
CN15
CN14
HRI8815Rt
HRI8806
HRI8450B
HRI8450A
HRI8506
HRI1279ARt
ATCC33913T, Rt
Xca5
LMG8004
HRI6382
HRI3849ARt
X. campestris pv. incanae HRI6378
X. campestris pv. armoraciae HRI6376
X. campestris HRI5219A
HRI8474
HRI6490Rt
HRI6519Rt
HRI6520
756C
HRI8305Rt
HRI8803Pt
HRI8298
HRI8299
X. oryzae pv. oryzae KACC10331
X. axonopodis pv. citri 306
X. euvesicatoria 85-10
A
B
C
0.01

**Figure 30**. (Previous page) Phylogenetic relationships among *Xanthomonas campestris* strains based on concatenated sequences of four genes (*dnaK*, *fyuA*, *rpoD* and *gyrB*). The phylogenetic tree was constructed by using the Maximum Likelihood method (Felsenstein, 1981) based on the Tamura-Nei model (Tamura & Nei, 1993). The percentage of trees in which the associated taxa clustered together is shown above the branches (bootstrap values). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 43 nucleotide sequences and 8,712 nucleotide positions, and it was conducted in MEGA6 software (Tamura *et al*., 2013). The strains of *X. oryzae* pv. *oryzae*, *X. axonopodis* pv. *citri* and *X. euvesicatoria* were used as an outgroup. Main clades are labelled as A, B and C; the clade comprising *X. campestris* pv. *campestris* strains is highlighted in green (clade A); the clade comprising *X. campestris* pv. *raphani* strains is highlighted in blue; clades comprising strains of the same race, which were consistent in different phylogenetic trees (this one and the tree presented in Figure 31) are highlighted in red. T, Pt and Rt, indicate type strains of species, pathovars and races, respectively.

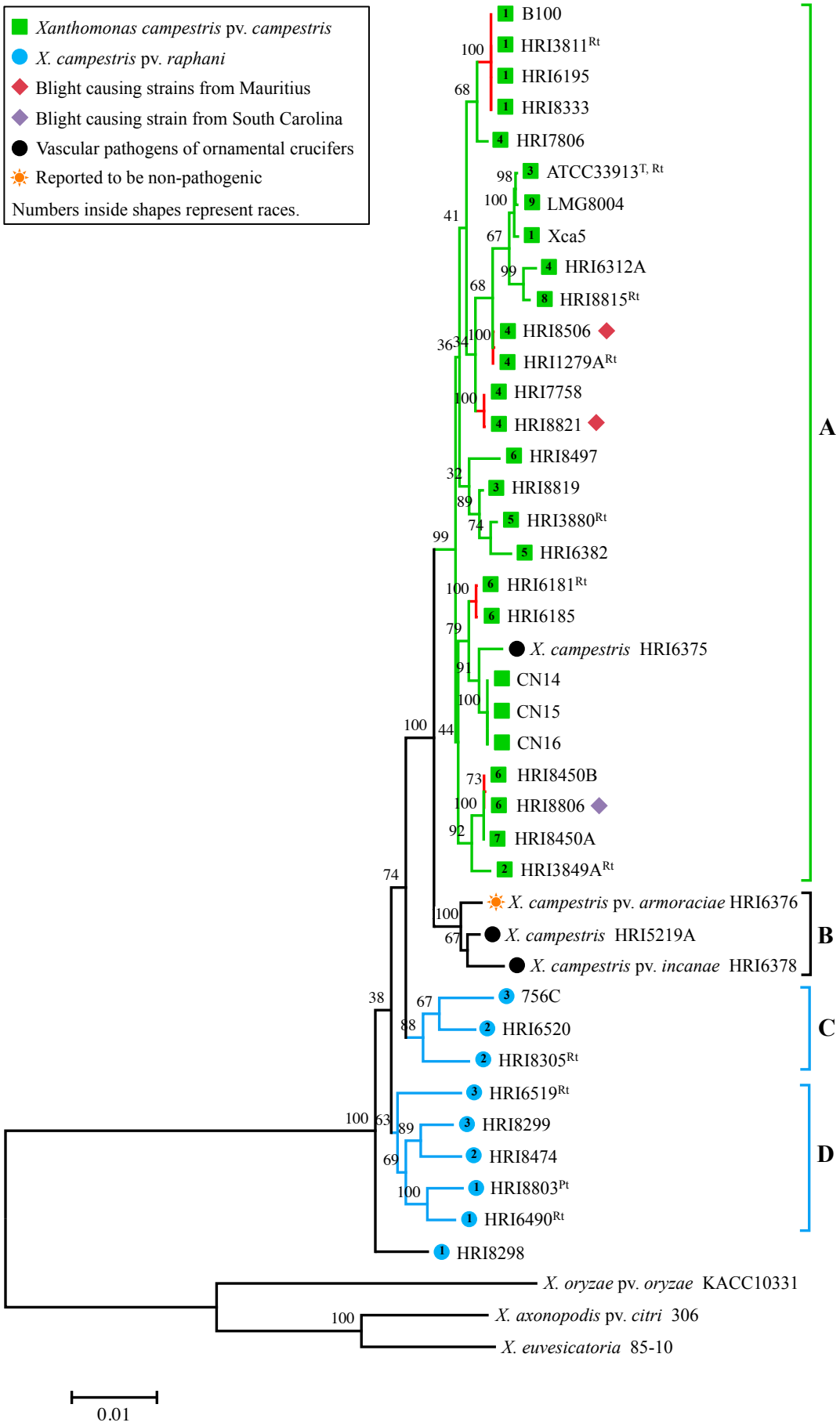
Xanthomonas campestris pv. campestris
X. campestris pv. raphani
Blight causing strains from Mauritius
Blight causing strain from South Carolina
Vascular pathogens of ornamental crucifers
Reported to be non-pathogenic
Numbers inside shapes represent races.
B100
HRI3811Rt
HRI6195
HRI8333
HRI7806
ATCC33913T, Rt
LMG8004
Xca5
HRI6312A
HRI8815Rt
HRI8506
HRI1279ARt
HRI7758
HRI8821
HRI8497
HRI8819
HRI3880Rt
HRI6382
HRI6181Rt
HRI6185
X. campestris HRI6375
CN14
CN15
CN16
HRI8450B
HRI8806
HRI8450A
HRI3849ARt
X. campestris pv. armoraciae HRI6376
X. campestris HRI5219A
X. campestris pv. incanae HRI6378
756C
HRI6520
HRI8305Rt
HRI6519Rt
HRI8299
HRI8474
HRI8803Pt
HRI6490Rt
HRI8298
X. oryzae pv. oryzae KACC10331
X. axonopodis pv. citri 306
X. euvesicatoria 85-10
A
B
C
D
0.01

**Figure 31**. (Previous page) Phylogenetic relationships among *Xanthomonas campestris* strains based on concatenated sequences of 31 genes. The set of genes used is described in Table 29 (Section 5.2.5). The phylogenetic tree was constructed by using the Maximum Likelihood method (Felsenstein, 1981) based on the General Time Reversible model (Nei & Kumar, 2000). The percentage of trees in which the associated taxa clustered together is shown above the branches (bootstrap values). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 43 nucleotide sequences and 23,379 nucleotide positions, and it was conducted in MEGA6 software (Tamura *et al*., 2013). The strains of *X. oryzae* pv. *oryzae*, *X. axonopodis* pv. *citri* and *X. euvesicatoria* were used as an outgroup. Main clades are labelled as A, B, C and D; the clade comprising *X. campestris* pv. *campestris* strains is highlighted in green (clade A); clades comprising *X. campestris* pv. *raphani* strains are highlighted in blue (clades C and D); clades comprising strains of the same race, which were consistent in different phylogenetic trees (this one and the tree presented in Figure 30) are highlighted in red. T, Pt and Rt, indicate type strains of species, pathovars and races, respectively.

### 5.3.2 Genome-wide identification of pathovar-associated genes

Genome-wide searches were performed to identify genes that are differentially present/absent between the strains assigned to *Xcr* and *Xcc* listed in Table 28 (Section 5.2.1). Following the criteria described in Materials and Methods (Section 5.2.6), five genes were predicted to be present in all *Xcr* strains and absent or highly divergent in all *Xcc* strains (*Xcr*-associated genes); and seven genes were predicted to be present in all *Xcc* strains and absent or highly divergent in all *Xcr* strains (*Xcc*-associated genes) (Table 30). Identification of each pathovar-associated gene was based primarily on the breadth of coverage from alignments of genomic sequence reads to a reference pan-genome that consisted of nucleotide sequences (pan-genes), each representing a cluster of highly similar gene sequences annotated in 32 *X. campestris* genomes. The breadth of coverage of each *Xcr*- and *Xcc*-associated pan-gene (that led to the identification of each gene) by genomic sequence reads of all strains under study, is presented in Appendix 15.

The *Xcr*-associated genes include a gene (*xopAD*) that is predicted to encode a T3E protein, and four additional genes predicted to encode a YapH protein (*Yersinia* autotransporter-like protein H), an alpha/beta hydrolase protein, a transcriptional regulator and a hypothetical protein. The *Xcc*-associated genes include four genes (*avrBs2, xopAM, xopK* and *xopQ*) that are each predicted to encode T3E proteins, and three additional genes predicted to encode a type I restriction enzyme M, an ATP-dependent DNA helicase and a hypothetical protein. The product of each gene was predicted based on the annotation of each reference gene sequence listed in Table 30 and sequence similarity to predicted/known T3E proteins in the RefSeq protein database (NCBI, http://www.ncbi.nlm.nih.gov) (Appendix 17) and in the *Xanthomonas* Resource database (http://www.xanthomonas.org/t3e.html) (Appendix 18). The genes identified are distributed across the reference complete genomes of *Xcr* strain 756C (RefSeq NC_017271.1) and *Xcc* strain ATCC33913 (RefSeq NC_003902.1) as shown in Figure 32.

**Table 30**. List of genes differentially present/absent (or highly divergent in sequence) in *Xanthomonas campestris* pv. *raphani* and *X. campestris* pv. *campestris*

| Gene/Pan-gene no. | Predicted gene product | Reference gene sequence | | | | | |
|---|---|---|---|---|---|---|---|
| | | Locus tag | RefSeq accession of genome assembly (location, bp) | Strain | Gene length (bp) | Predicted gene product from genome annotation | Similarity to predicted/known type III effector proteins in the RefSeq protein database and/or the *Xanthomonas* Resource database [a] |
| ***Xcr*-associated genes** | | | | | | | |
| 1 | XopAD (type III effector) | XCR_1464 | NC_017271.1 (1,488,733-1,495,629) | 756C | 6897 | Type III effector protein XopAD | XopAD |
| 2 | YapH protein (*Yersinia* autotransporter-like protein H) | XCR_0904 | NC_017271.1 (944,768-948,478) | 756C | 3711 | YapH protein, putative | nd |
| 3 | Alpha-beta hydrolase | * | na, this study | HRI6519 | 1029 | Alpha-beta hydrolase | nd |
| 4 | Transcriptional regulator (TetR family) | * | na, this study | HRI6519 | 642 | Transcriptional regulator (TetR family) | nd |
| 5 | Hypothetical protein | * | na, this study | HRI8474 | 333 | Hypothetical protein | nd |
| ***Xcc*-associated genes** | | | | | | | |
| 6 | AvrBs2 (type III effector) | XC_0052 | NC_007086.1 (64,947-67,103) | LMG8004 | 2157 | Avirulence protein | AvrBs2 |
| 7 | XopAM (type III effector) | XCC1089 | NC_003902.1 (1,258,786-1,264,935) | ATCC33913 | 6150 | Transducer protein car | XopAM |
| 8 | XopK (type III effector) | xccb100_1254 | NC_010688.1 (1,447,627-1,450,200) | B100 | 2574 | Hypothetical protein | XopK |
| 9 | XopQ (type III effector) | XCC1072 | NC_003902.1 (1,237,985-1,236,606) | ATCC33913 | 1380 | Hypothetical protein | XopQ |
| 10 | Type I restriction enzyme M | XCC2902 | NC_003902.1 (3,442,864-3,444,372) | ATCC33913 | 1509 | Type I restriction enzyme M | nd |
| 11 | ATP-dependent DNA helicase | XC_0537 | NC_007086.1 (637,335-638,651) | LMG8004 | 1317 | ATP-dependent DNA helicase | nd |
| 12 | Hypothetical protein | XCC1073 | NC_003902.1 (1,238,424-1,239,173) | ATCC33913 | 750 | Hypothetical protein | nd |

Abbreviations: nd, not detected; no., number; *Xcc*, *Xanthomonas campestris* pv. *campestris*; *Xcr*, *X. campestris* pv. *raphani*.

[a] Similarity searches were performed by BLASTX against the RefSeq protein database (NCBI, www.ncbi.nlm.nih.gov) (see Appendix 17) and predicted/known type III effector protein sequences from whole genome sequenced strains of *Xanthomonas* spp. (listed in the *Xanthomonas* Resource database, http://www.xanthomonas.org/t3e.html) (see Appendix 18).

* Gene sequence provided in Appendix 16.

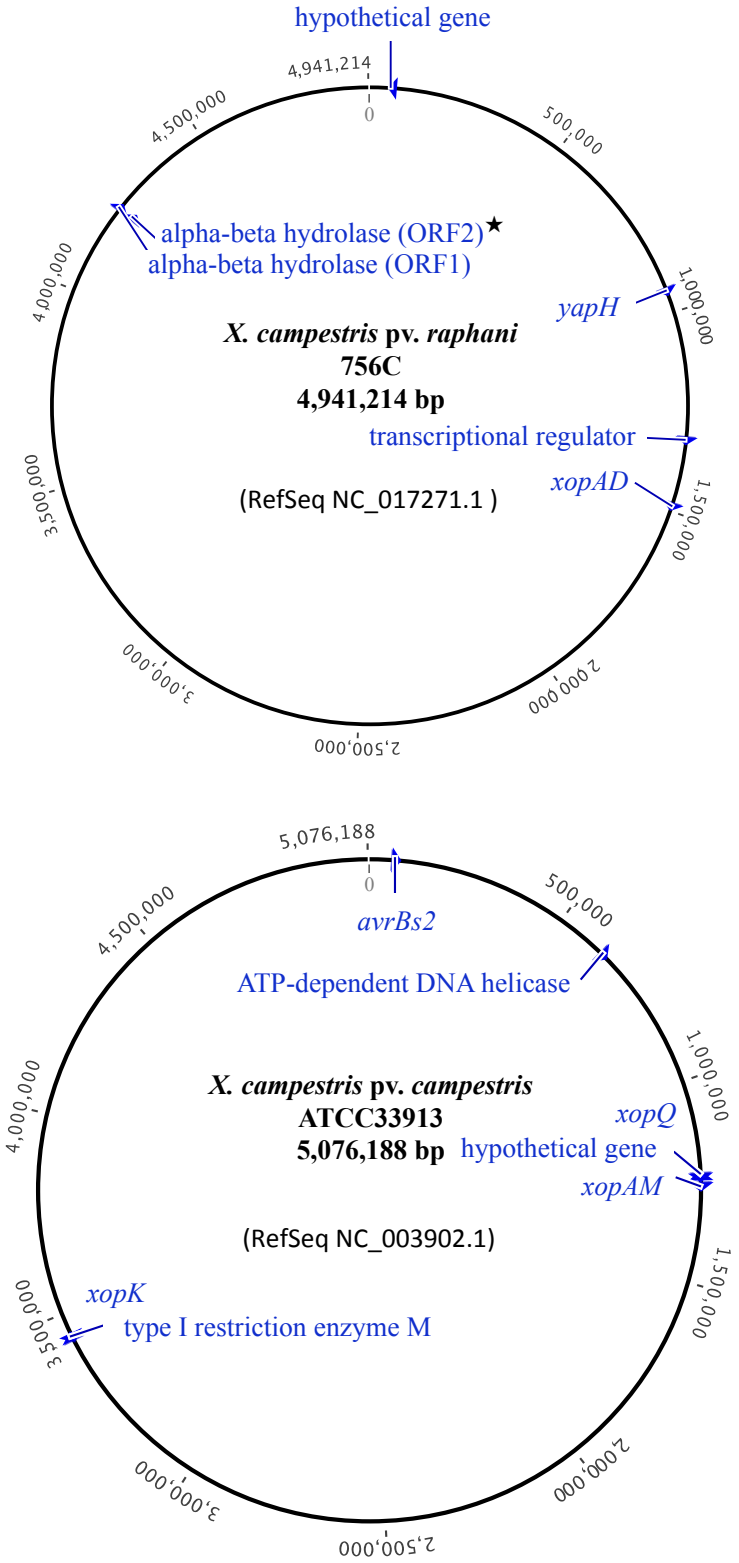


**Figure 32**. Diagram of the relative position of genes identified in *Xanthomonas campestris* pv. *raphani* and not detected in pv. *campestris* (top) or identified in pv. *campestris* and not detected in pv. *raphani* (bottom), on the chromosome maps of *X. campestris* pv. *raphani* strain 756C and *X. campestris* pv. *campestris* strain ATCC33913, respectively. ★The corresponding sequence to the full-length alpha-beta hydrolase gene (1029 bp) is split into two neighboring open reading frames (ORF1 and ORF2), each including an annotated locus predicted to encode an alpha-beta hydrolase (see text).

For each pathovar-associated reference gene sequence listed in Table 30, a corresponding coding sequence was identified in the genome assemblies of the remaining strains of the respective pathovar, further confirming the presence of each gene in those strains. All sequences encoded amino acid sequences that shared high identity with the translated reference sequence as shown in Tables 31 and 32, and all had similar length to the reference sequence except in two cases. The sequence of the *Xcr*-associated gene 2 (predicted to encode a YapH protein) identified in strain HRI8474 (length 3,282 bp) was 429 bp shorter than the reference sequence of strain 756C (length 3,711 bp). In the case of the *Xcr*-associated gene 3 (predicted to encode an alpha-beta hydrolase protein), a coding sequence of identical length to the reference sequence of this gene was identified in all *Xcr* strains (length 1,029 bp) except in the complete genome sequence of *Xcr* strain 756C (RefSeq accession NC_017271). In this genome sequence, the corresponding sequence is split into two neighbouring open reading frames (ORF1 of length 465 bp and ORF2 of length 567 bp), each sharing high identity with the respective aligned fragment of the reference protein sequence (69.5% identity for ORF1 and 98.9% identity for ORF2; Table 31, Figure 33). Each of these ORFs comprise an annotated locus predicted to encode an alpha-beta hydrolase protein (loci XCR_3954 and XCR_3955), suggesting that one or two alpha-beta hydrolase proteins might be expressed in strain 756C.

Four *X. campestris* strains with distinct pathogenic features from *Xcr* and *Xcc* strains were included in this study (three vascular pathogens each from a different ornamental crucifer, and a strain identified as *X. campestris* pv. *armoraciae*, but that has been reported to be non-pathogenic in several *Brassicaceae* plants). Some *Xcr*- and *Xcc*-associated genes were also detected in each of these strains by high breadth of coverage by genomic sequence reads (as shown in Appendix 15). However, the full set of either the *Xcr*-associated or the *Xcc*-associated genes was not detected by high breadth of coverage in any of these strains.

**Table 31**. Percentage of amino acid sequence identity of *Xanthomonas campestris* pv. *raphani*-associated gene products compared with a selected reference sequence (R)

| Strain | | Predicted gene product (gene/pan-gene no.) | | | | |
|---|---|---|---|---|---|---|
| Race | Accession | XopAD | YapH | Alpha-beta hydrolase | Transcriptional regulator | Hypothetical protein |
| | | (1) | (2) | (3) | (4) | (5) |
| 1 | HRI6490 | 99.8 | 99.0 | 98.2 | 97.2 | 95.5 |
| 1 | HRI8298 | 99.8 | 99.0 | 99.1 | 97.7 | 90.0 |
| 1 | HRI8803 | 99.8 | 98.4 | 99.1 | 97.7 | 86.4 |
| 2 | HRI6520 | 100.0 | 99.0 | 98.8 | 97.2 | 97.3 |
| 2 | HRI8305 | 99.9 | 99.1 | 99.4 | 97.7 | 90.9 |
| 2 | HRI8474 | 99.9 | 97.6 [b] | 97.4 | 99.1 | R |
| 3 | HRI6519 | 99.7 | 97.8 | R | R | 90.9 |
| 3 | HRI8299 | 100.0 | 98.5 | 98.8 | 98.1 | 95.5 |
| 3 | 756C [a] | R | R | 69.5 ORF1 [c] 98.9 ORF2 [c] | 96.7 | 93.6 |

Color key

0 100

[a] Strain with published complete genome sequence (Bogdanove *et al.*, 2011).

[b] The predicted protein sequence is 143 aa shorter (total length of 1093 aa) than the reference protein sequence (total length of 1236 aa).

[c] The matching sequence to the full-length reference gene sequence is split into two neighbouring open reading frames (ORF1 and ORF2) predicted to encode two smaller protein sequences (154 aa and 188 aa, respectively) than the reference protein sequence (342 aa) (see also Figure 32).

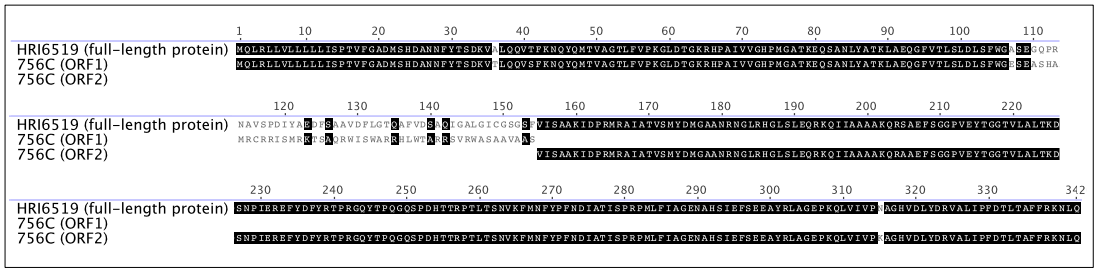


**Figure 33**. Alignment of the full-length alpha-beta hydrolase protein sequence from *Xanthomonas campestris* pv. *raphani* strain HRI6519 with the protein sequences of two neighbouring open reading frames (ORF1 and ORF2) identified in strain 756C. Black shades highlight identical amino acids.

**Table 32**. Percentage of amino acid sequence identity of *Xanthomonas campestris* pv. *campestris*-associated gene products compared with a selected reference sequence (R)

| Strain | | Predicted gene product (gene/pan-gene no.) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Race | Accession | AvrBs2 | XopAM | XopK | XopQ | Type I restriction enzyme M | ATP-dependent DNA helicase | Hypothetical protein |
| | | (1) | (2) | (3) | (4) | (5) | (6) | (7) |
| 1 | 3811 | 99.9 | 100.0 | 100.0 | 99.6 | 100.0 | 100.0 | 100.0 |
| 1 | 6195 | 99.9 | 100.0 | 99.5 | 99.6 | 100.0 | 100.0 | 100.0 |
| 1 | 8333 | 99.9 | 100.0 | 100.0 | 99.6 | 100.0 | 100.0 | 100.0 |
| 1 | B100 [a] | 99.9 | 100.0 | R | 99.6 | 100.0 | 100.0 | 100.0 |
| 2 | 3849A | 99.9 | 100.0 | 99.5 | 99.6 | 98.4 | 100.0 | 100.0 |
| 3 | 8819 | 99.9 | 100.0 | 99.8 | 99.6 | 100.0 | 99.8 | 100.0 |
| 3 | ATCC33913 [a] | 100.0 | R | 99.8 | R | R | 100.0 | R |
| 4 | 1279A | 99.9 | 100.0 | 100.0 | 99.8 | 97.2 | 99.8 | 100.0 |
| 4 | 6312A | 100.0 | 100.0 | 100.0 | 99.6 | 99.8 | 100.0 | 100.0 |
| 4 | 7758 | 99.9 | 100.0 | 99.6 | 99.6 | 98.6 | 95.2 | 100.0 |
| 4 | 7806 | 99.9 | 100.0 | 100.0 | 99.6 | 97.2 | 100.0 | 100.0 |
| 4 | 8506 | 99.9 | 100.0 | 100.0 | 99.8 | 97.2 | 99.8 | 100.0 |
| 4 | 8821 | 100.0 | 100.0 | 99.6 | 99.8 | 98.6 | 95.2 | 100.0 |
| 5 | 3880 | 99.9 | 99.9 | 99.8 | 100.0 | 100.0 | 99.8 | 100.0 |
| 5 | 6382 | 99.9 | 100.0 | 99.8 | 99.6 | 100.0 | 99.8 | 100.0 |
| 6 | 6181 | 99.9 | 100.0 | 99.8 | 99.6 | 100.0 | 99.5 | 100.0 |
| 6 | 6185 | 99.9 | 100.0 | 99.8 | 99.6 | 100.0 | 99.8 | 100.0 |
| 6 | 8450B | 99.9 | 100.0 | 99.8 | 99.8 | 100.0 | 100.0 | 98.8 |
| 6 | 8497 | 99.9 | 99.9 | 99.6 | 99.6 | 100.0 | 99.5 | 100.0 |
| 6 | 8806 | 99.9 | 100.0 | 99.8 | 99.8 | 100.0 | 100.0 | 98.8 |
| 7 | 8450A | 99.9 | 100.0 | 100.0 | 99.8 | 100.0 | 100.0 | 98.8 |
| 8 | 8815 | 100.0 | 100.0 | 99.8 | 100.0 | 100.0 | 100.0 | 100.0 |
| 9 | LMG8004 [a] | R | 100.0 | 99.8 | 99.6 | 100.0 | R | 98.4 |

Color key


[a] Strains with published complete genome sequences: ATCC33913 (da Silva *et al.*, 2002); B100 (Vorhölter *et al.*, 2008); LMG8004 (Qian *et al.*, 2005); 756C (Bogdanove *et al.*, 2011).

### 5.3.3 Genome-wide identification of candidate avirulence genes of races of *Xanthomonas campestris* pathovars *raphani* and *campestris*

Genome-wide searches focused on identifying candidates for avirulence genes that are determinants of races of *Xcr* and *Xcc* as predicted in the gene-for-gene models summarized in Table 2 (Chapter 1). Searches were performed according to the presence/absence patterns of avirulence genes predicted in different races as shown in Table 27 (Section 5.1) and following the criteria presented in Materials and Methods (Section 5.2.6) or with the modifications described below. The genes identified are listed in Table 33 and a reference gene sequence is given.

For avirulence genes predicted in *Xcr* races, two candidates were identified for the *A1r* gene (present in race 1 and absent from races 2 and 3) and one candidate was identified for the *A2r* gene (present in race 2 and absent from races 1 and 3). The *A1r* candidates include a gene predicted to encode for a component of an ATP-binding cassette (ABC) transporter (gene 13) and a hypothetical protein (gene 14) that showed homology to an unnamed T3E protein from *Ralstonia solanacearum* in BLASTX searches against the RefSeq protein database (query coverage 72%; sequence identity 36%; Appendix 20). The candidate identified for the *A2r* gene (present in race 2 and absent from races 1 and 3) is predicted to encode a T3E protein (XopG, syn. HopH1) based on the results from BLASTX searches against the RefSeq database (Appendix 20) and predicted/known T3E proteins reported from *Xanthomonas* spp. (Appendix 21; http://www.xanthomonas.org/t3e.html).

In *Xcc*, no candidates were found for three avirulence genes (*A1c*, *A3c* and *A4c*) when searches were performed using the breadth of coverage (BC) thresholds, >80% for presence and <20% for absence. However, in an attempt to mine the data for candidates for these avirulence genes, the criteria described in Materials and Methods (Section 5.2.6) were modified by applying less stringent BC thresholds as follows: BC >80% for presence of a gene, but allowing BC ≤80% for at most one strain where each avirulence gene is predicted to be present; and BC <30% for absence or high divergence in sequence (instead of BC <20%). When searches were conducted using these less stringent BC thresholds, one candidate was identified for each of the avirulence genes *A1c* and *A3c* (Table 33), but no candidates were found for *A4c*.

The *A1c* candidate gene (gene 16 in Table 33) is predicted to encode an oxidoreductase protein that shared sequence similarity with short-chain dehydrogenase/reductase proteins in the RefSeq database (Appendix 20). The predicted gene product did no show sequence similarity to any predicted/known T3E proteins in RefSeq protein database or in the *Xanthomonas* Resource database (Appendixes 20 and 21). This gene was identified in strains of all races predicted to have the *A1c* avirulence gene (races 1, 3, 4, 8 and 9) except for one strain of race 3 (HRI8819) and absent (or highly divergent) in strains of the remaining races (Table 34). For strain HRI8819, the BC of the corresponding pan-gene by genomic reads of this strain was 0% (Appendix 19) and BLASTN searches against the genome assemblies of this strain (*de novo* assembly and reference-guided assembly) resulted in one hit with query coverage 22.4%. These results suggest that the gene is either absent from this strain or is at least highly divergent in sequence.

The candidate identified for the *Xcc A3c* avirulence gene corresponds to the same candidate gene identified for the *Xcr* avirulence gene *A2r*. It is present in strains of all *Xcc* races predicted to have the *A3c* gene except one race (*i.e.* races 3, 5, 8 and 9 except race 2) and absent (or highly divergent) in strains of the remaining races (Table 34). Only one strain of race 2 (HRI3849A) was included in this study. The BC of the corresponding pan-gene by genomic reads of this strain was 0% (Appendix 19) and BLASTN searches against the genome assembly of this strain resulted in no hits suggesting that the gene is absent or highly divergent in sequence in this strain.

For each reference gene sequence listed in Table 33, a corresponding coding sequence was identified in the genome assemblies of the remaining strains where presence of each gene was predicted based on BC values, confirming the presence of each gene in those strains. All sequences encoded amino acid sequences that shared high identity with the translated reference sequence (Table 34) and all had identical length except in one case. The *xopG* gene is annotated as 294 bp long in the published complete genome of strain LMG8004 (RefSeq accession NC_007086.1, locus tag XC_0967) whereas in the remaining strains, the coding sequence was 624 bp long.

**Table 33**. List of candidates for avirulence genes of races of *Xanthomonas campestris* pv. *raphani* and *X. campestris* pv. *campestris* [a]

| Gene/Pan-gene no. | Predicted gene product | Reference gene sequence | | | | | |
|---|---|---|---|---|---|---|---|
| | | Locus tag | RefSeq accession of genome assembly (location, bp) | Strain | Gene length (bp) | Predicted gene product from genome annotation | Similarity to predicted/known type III effectors proteins in the RefSeq protein database and/or the *Xanthomonas* Resource database [b] |
| **Candidates for the avirulence gene *A1r* (present in *Xcr* race 1; absent from remaining *Xcr* races)** | | | | | | | |
| 13 | ABC transporter | * | na, this study | HRI8803 | 750 | ATP-binding cassette (ABC) transporter | nd |
| 14 | Hypothetical protein | * | na, this study | HRI8803 | 420 | Hypothetical protein | Type III effector (*Ralstonia solanacearum*) |
| **Candidates for the avirulence genes *A2r* (present in *Xcr* race 2; absent from remaining *Xcr* races) and *A3c* (present in *Xcc* races 3, 5, 8 and 9; absent from remaining *Xcc* races)** | | | | | | | |
| 15 | XopG (type III effector) | XCC3258 | NC_003902.1 (3,871,675-3,871,052) | ATCC33913 | 624 | Hypothetical protein | XopG (syn. HopH1) |
| **Candidate for the avirulence gene *A1c* (present in *Xcc* races 1, 3, 4, 8, 9 except for one strain of race 3; absent from remaining *Xcc* races)** | | | | | | | |
| 16 | Oxidoreductase | * | na, this study | HRI3811 | 942 | 3-oxoacyl-[acyl-carrier protein] reductase (oxidoreductase family) | nd |

Abbreviations: nd, not detected; no., number; *Xcc*, *X. campestris* pv. *campestris*; *Xcr*, *X. campestris* pv. *raphani*.

[a] Candidates for avirulence genes predicted in the published gene-for-gene models to explain the interactions between races of each pathovar and *Brassicaceae* differentials (summarized in Table 2, Chapter 1; or Table 27, Section 5.1 of the present Chapter).

[b] Similarity searches were performed by BLASTX against the RefSeq protein database (NCBI, www.ncbi.nlm.nih.gov) (see Appendix 20) and predicted/known type III effector proteins from whole genome sequenced strains of *Xanthomonas* spp. (listed in the *Xanthomonas* Resource database, http://www.xanthomonas.org/t3e.html) (see Appendix 21).

* Gene sequence provided in Appendix 16.

**Table 34**. Presence/absence of candidate avirulence genes across strains of races of *Xanthomonas campestris* pathovars *raphani* and *campestris*, and percentage of amino acid sequence identity of each predicted gene product with a selected reference sequence (R) [a]

| Strain | | Predicted gene product (avirulence gene; gene/pan-gene no.) | | | |
|---|---|---|---|---|---|
| Race | Accession | ABC transporter (*A1r*; 13) | Hypothetical protein (*A1r*; 14) | XopG (*A2r* and *A3c*; 15) | Oxidoreductase (*A1c*; 16) |
| ***Xanthomonas campestris* pv. *raphani*** | | | | | |
| 1 | HRI6490 | + (99.6) | + (97.8) | − | |
| 1 | HRI8298 | + (100.0) | + (100.0) | − | |
| 1 | HRI8803 | **+ (R)** | **+ (R)** | − | |
| 2 | HRI6520 | − | − | + (99.5) | |
| 2 | HRI8305 | − | − | + (99.5) | |
| 2 | HRI8474 | − | − | + (100.0) | |
| 3 | HRI6519 | − | − | − | |
| 3 | HRI8299 | − | − | − | |
| 3 | 756C [b] | − | − | − | |
| ***X. campestris* pv. *campestris*** | | | | | |
| 1 | HRI3811 | | | − | **+ (R)** |
| 1 | HRI6195 | | | − | + (100.0) |
| 1 | HRI8333 | | | − | + (100.0) |
| 1 | B100 [b] | | | − | + (99.7) |
| 2 | HRI3849A | | | − | − |
| 3 | HRI8819 | | | + (100.0) | − |
| 3 | ATCC33913 [b] | | | **+ (R)** | + (100.0) |
| 4 | HRI1279A | | | − | + (100.0) |
| 4 | HRI6312A | | | − | + (100.0) |
| 4 | HRI7758 | | | − | + (100.0) |
| 4 | HRI7806 | | | − | + (100.0) |
| 4 | HRI8506 | | | − | + (100.0) |
| 4 | HRI8821 | | | − | + (100.0) |
| 5 | HRI3880 | | | + (100.0) | − |
| 5 | HRI6382 | | | + (100.0) | − |
| 6 | HRI6181 | | | − | − |
| 6 | HRI6185 | | | − | − |
| 6 | HRI8450B | | | − | − |
| 6 | HRI8497 | | | − | − |
| 6 | HRI8806 | | | − | − |
| 7 | HRI8450A | | | − | − |
| 8 | HRI8815 | | | + (100.0) | + (100.0) |
| 9 | LMG8004 [b] | | | + (99.0) [c] | + (100.0) |

Symbols: +, gene present; −, gene absent or highly divergent in sequence, based on the breadth of coverage from alignments of genomic sequence reads with a pan-gene sequence representing each gene (maximum breadth of coverage 25.2%; see Appendix 19) and BLAST hits of each pan-gene sequence against the genome assemblies determined for each strain (maximum query coverage 25% at any level of sequence identity).

[a] The percentage of amino acid sequence identity of each predicted gene product with a selected reference sequence (R) is given in brackets; grey shade indicates not determined presence/absent of each gene.

[b] Strains with published complete genomes: ATCC33913 (da Silva *et al.*, 2002); B100 (Vorhölter *et al.*, 2008); LMG8004 (Qian *et al.*, 2005); 756C (Bogdanove *et al.*, 2011).

[c] The predicted protein is 110 aa shorter than the reference protein sequence (207 aa).

## 5.4 DISCUSSION

**Phylogenetic relatedness of *Xanthomonas campestris* strains under study**

All of the *Xcc* strains in this study are more closely related to each other than they are to most of the remaining strains compared including the leaf spotting *Xcr* strains, as they grouped in a single clade with high bootstrap support in both phylogenetic trees generated in this study (Figures 30 and 31). The *X. campestris* strain from candytuft (HRI6375) also grouped together with *Xcc* strains similarly to the results obtained in previous studies (Vicente *et al.*, 2006; Fargier *et al.*, 2011). This strain causes vascular symptoms in candytuft similar to those caused by two *Xcc* strains in this host (Fargier & Manceau, 2007), but it differs from typical *Xcc* strains because it has only been described to be pathogenic in candytuft and radish but not in brassicas including the differentials of *Xcc* races (Vicente *et al.*, 2001; Vicente *et al.*, 2006; Fargier & Manceau, 2007).

In comparison to *Xcc* strains, the two vascular pathogens of ornamental crucifers (the *X. campestris* pv. *incanae* strain from garden stock and a strain from wallflower) and the *X. campestris* pv. *armoraciae* strain (which has been reported to be non-pathogenic) also grouped in a single clade supported by high bootstrap in both phylogenetic trees. However, the genetic relatedness of the *Xcr* strains was less clear because clades were not consistent in both phylogenetic trees and some had low bootstrap support, despite a tendency for *Xcr* strains to group together. Interestingly, the branch lengths were considerably long for the *Xcr* strains suggesting that there is a high level of genetic divergence within *Xcr* as previously reported by Fargier *et al.* (2011). Further resolution of phylogenetic relatedness among the strains may be possible for example by genome-wide analysis of single nucleotide polymorphisms as performed by Baltrus *et al.* (2014) for phylogenetic analysis of *Pseudomonas syringae* pv. *pisi* strains.

Overall, the phylogenetic analyses presented in this study showed separation between strains of *Xcr* and *Xcc* that cause distinct disease symptoms in common hosts within the *Brassicaceae* family, and *Xcr* has also a wider host range than *Xcc*, which includes tomato from the *Solanaceae* family (Bradbury, 1986; Vicente *et al.*, 2006; Fargier & Manceau, 2007). These results are in agreement with previous studies that assessed the genetic relatedness of *X. campestris* pathovars including *Xcr*

and *Xcc*, based on repetitive sequence-based PCR genomic fingerprinting (Vicente *et al*., 2006) and housekeeping genes (Fargier *et al.*, 2011). Given these results, it is tempting to speculate that adaptation to different modes of pathogenesis and/or host range may have contributed to the evolutionary trajectories of these pathovars. Regarding races within these pathovars, although some *Xcc* strains of the same race grouped together with high bootstrap support, the phylogenetic analyses indicate that races are not strictly correlated with phylogenetic groups as shown in previous studies (Vicente *et al.*, 2006; Fargier *et al.*, 2011). Races are distinguished based on differential interactions with specific plant host genotypes and if genes conferring race specificity are acquired by horizontal gene transfer, then correlation between races and phylogenetic groups would not be expected.

**Candidate determinants of distinct pathogenic features of *Xanthomonas campestris* pathovars *raphani* and *campestris***

Genome-wide searches were performed in this study to identify genes that are differentially present/absent (or highly divergent) between *Xcr* and *Xcc* strains, and which may contribute to their distinct pathogenic features. Searches were performed primarily based on breadth of coverage from alignments of genomic sequence reads to a pan-genome composed of nucleotide sequences (pan-genes), each representing highly similar gene sequences from 32 *X. campestris* genomes. Importantly, these searches were performed without applying prior knowledge of predicted biological functions. The genes identified include genes that have been described to have an effect on pathogenicity, as well as other genes for which a function related to pathogenicity has not yet been reported.

Many bacterial pathogens including *Xanthomonas* spp. use the type III secretion system to deliver effector (T3E) proteins inside the plant cells, which is essential for pathogenicity (Büttner & Bonas, 2010). Specific T3E proteins have been reported to suppress plant immunity and manipulate plant cellular processes creating favorable conditions for pathogen growth and spread (Grant *et al.*, 2006; White *et al.*, 2009; Macho, 2016). Among the genes identified in this study as differentially present/absent (or highly divergent) between nine *Xcr* and 23 *Xcc* strains, five genes are predicted to encode T3E proteins including *xopAD* which is present in *Xcr*, and *avrBs2*, *xopAM*, *xopK* and *xopQ* which are present in *Xcc*.

Bogdanove *et al.* (2011) reported the presence of *xopAD* in the reference genome of *Xcr* strain 756C; however, the role of the predicted T3E protein in pathogenicity of *Xcr* is unknown. Among the four T3E genes identified in *Xcc*, three have been reported to have an effect on pathogenicity. The genes *avrBs2*, *xopAM* (or *xopR1*) and *xopQ* have been shown to be individually required for full virulence of *Xcc* strain LMG8004 in Chinese radish (Xu *et al.*, 2006; Jiang *et al.*, 2009). *xopAM* has also been reported to confer partial avirulence to that strain in the *A. thaliana* accession Col-0 (Guy *et al.*, 2013). The contribution of *xopK* to pathogenicity of *Xcc* is unknown (Vicente & Holub, 2013) and mutagenesis of the *xopK* gene from strain LMG8004 did not affect pathogenicity in *A. thaliana* (Guy *et al.*, 2013).

Other T3E genes have also been reported as differentially present/absent between *Xcr* and *Xcc* strains (Bogdanove *et al.*, 2011; Roux *et al.*, 2015). The previous reports only included one or two *Xcr* strains for comparison with a few *Xcc* strains. These genes were not identified in the current study possibly because the larger sample of strains compared may include strains of both pathovars that contain those genes or share close homologues. For example, *xopN* has been identified in *Xcc*, but not in *Xcr* (Bogdanove *et al.*, 2011; Roux *et al.*, 2015). However, *xopN* was identified in all *Xcc* strains as well as two *Xcr* strains (HRI8305 and 6520) compared in this study (data not shown).

Another gene identified in *Xcr* strains, but not in *Xcc* strains, is a gene predicted to encode an adhesin-like protein YapH (*Yersinia* autotransporter-like protein H). Adhesins are proteinaceous structures in the surface of bacterial cells that facilitate bacterial attachment to surfaces and colonization of host tissues (Büttner & Bonas, 2010; Ryan *et al.*, 2011). In particular, YapH homologues have been reported to be involved in virulence and/or attachment to plant tissues in *X. oryzae* pv. *oryzae* and *X. fuscans* subsp. *fuscans* (Darsonval *et al.*, 2009; Das *et al.*, 2009). However, to date, the role of YapH homologues in *X. campestris* is unknown.

An association to pathogenicity of the remaining genes identified is unclear based on their predicted gene products (alpha-beta hydrolase, transcriptional regulator, hypothetical protein, type I restriction enzyme M and ATP-dependent DNA helicase) and sequence similarity to proteins in the RefSeq database or T3E proteins from *Xanthomonas* spp. listed in the *Xanthomonas* Resource database (http://www.xanthomonas.org/t3e.html). However, the fact that three out of seven

genes identified in *Xcc* strains have already been reported to have an effect on pathogenicity, supports the expectation that at least some other genes identified may also have a role in pathogenicity, especially genes identified in *Xcr*. Even though several pathogenicity factors have been identified in *Xcc* (Alvarez, 2000; Ryan *et al.*, 2011; Vicente & Holub, 2013), the pathogenicity factors of *Xcr* are poorly characterized and none of the genes identified in *Xcr* have been previously studied. Moreover, three T3E genes identified in *Xcc*, but not in *Xcr*, have only been shown to contribute to virulence of a specific *Xcc* strain (LMG8004) (Xu *et al.*, 2006; Jiang *et al.*, 2009) and it is not known whether they play a key role in pathogenesis of the pathovar. The short list of genes identified as differentially present/absent (or highly divergent) between multiple strains of *Xcr* and *Xcc* provides an excellent focus for future experiments to determine whether these genes (individually or collectively) play a key role in the distinct modes of pathogenesis of these pathovars. For example, do individual genes or a repertoire of these genes enable *Xcc* to colonize the vascular system, or restrict *Xcr* to the parenchyma tissues in the same host?

To date, biochemical or genetic markers have not been developed to delineate pathovars within *X. campestris sensu stricto*. The complete set of genes identified in either *Xcr* or *Xcc* strains was not detected in any of the four additional *X. campestris* strains that were included in this study and have distinct pathogenic features from *Xcr* and *Xcc*. Therefore, the sets of genes identified could potentially be used to develop pathovar-specific markers to assist pathogen diagnostics. This would require testing whether these genes are present in additional strains of *X. campestris.*

Interestingly, a higher proportion of genes that were identified in *Xcc*, but not in *Xcr*, was also detected in the vascular pathogens of ornamental crucifers in comparison to the genes identified in *Xcr*, but not in *Xcc*. Some of these genes may therefore play a role in the vascular behaviour of *X. campestris* pathogens. Another interesting observation is that the vascular pathogen of candytuft (HRI6375) is closely related to *Xcc* strains as observed in the phylogenetic analyses, but differs from typical *Xcc* strains because it has been reported to be non-pathogenic in several brassicas tested including the host differentials of *Xcc* races (Vicente *et al.*, 2001; Vicente *et al.*, 2006; Fargier & Manceau, 2007). This strain also differs from the *Xcc* strains compared in this study because it does not have the *avrbs2* gene (or has a

highly divergent gene sequence) whereas this gene is one of the core T3E genes of *Xcc* (Guy *et al.*, 2013; Roux *et al.*, 2015).

**Candidate avirulence determinants of races defined within *X. campestris* pathovars *raphani* and *campestris***

Genes conferring avirulence to *Xcr* races have not yet been identified. In *Xcc*, a few genes have been reported to confer avirulence in *Brassica* genotypes, but these genes have been identified in a restricted number of strains and have not yet been linked to the proposed gene-for-gene model of *Xcc* races (Vicente & Holub, 2013). In the present study, genome-wide searches identified candidate genes for two avirulence determinants predicted in the published gene-for-gene model of *Xcr* races (*A1r* and *A2r*, Table 27), and two avirulence determinants predicted in the gene-for-gene model of *Xcc* races (*A1c* and *A3c*, Table 27). None of the genes identified has been previously associated to avirulence in *X. campestris*.

In *Xcr*, two candidates were identified for the avirulence gene *A1r* (present in race 1; absent or highly divergent in strains of races 2 and 3). One candidate gene is predicted to encode a component of an ABC transporter and no avirulence function can be predicted from this annotation. Many avirulence proteins identified in plant pathogenic bacteria have been confirmed to be T3E proteins (Alfano & Collmer, 2004; Grant *et al.*, 2006; Mansfield, 2009). The predicted product of the second gene showed sequence similarity to a T3E protein that has been predicted from the genome sequence of a *Ralstonia solanacearum* strain (Ramesh *et al.*, 2014). This gene may encode a yet undescribed T3E that is recognized in the differential plant lines resistant to strains of *Xcr* race 1, specifically *B. juncea* line FBLM2, *B. rapa* cv. Just Right Hybrid Turnip and *B. napus* line Cob60 (Vicente *et al.*, 2006; Table 2, Chapter 1).

Interestingly, one candidate avirulence gene was identified in races of both pathovars, suggesting that they may share a common avirulence determinant. A candidate for the *A2r* gene was identified in strains of *Xcr* race 2, and the same gene was also identified in strains of *Xcc* races 3, 5, 8 and 9 (as a candidate for the avirulence gene *A3c*). The matching *R*-gene for *A2r* and *A3c* is most likely contained in the differential *B. oleracea* cv. Miracle, which is resistant to all these

*Xcr* and *Xcc* races (Table 2, Chapter 1). This avirulence candidate gene is predicted to encode a T3E protein designated XopG (syn. HopH1; White *et al.*, 2009; Potnis *et al.*, 2011). This is the first report of *xopG* in *Xcr* strains. The two *Xcr* strains for which there is a publicly available genome sequence, strains 756C (Bogdanove *et al.*, 2011) and CFBP5828 (Roux *et al.*, 2015) have been assigned to race 3 (Fargier & Manceau, 2007) and do not have this gene.

In *Xcc*, the *xopG* gene has been identified in several strains (White *et al.*, 2009; Guy *et al.*, 2013; Roux *et al.*, 2015), but its role in pathogenicity of *Xcc* has not been studied. The predicted products of the *xopG* alleles of *Xcr* and *Xcc* strains were of identical length (207 aa) and shared high homology with the reference sequence from the *Xcc* strain ATCC33913 (99.5 or 100%) except in one case. The *xopG* allele from the *Xcc* race 9 strain LMG8004, is truncated and the predicted gene product is only 97 aa long. However, it is not known whether this gene is still functional. Furthermore, the *xopG* gene was not identified in the only strain of *Xcc* race 2 included in this study, which is also predicted to have the *A3c* gene in the gene-for-gene model (Table 2, Chapter 1). If this gene is confirmed to be an avirulence determinant in *B. oleracea* cv. Miracle, another gene would be required to explain avirulence of race 2 in this host differential.

Finally, an *A1c* candidate was identified which is present in all strains of *Xcc* races 1, 3, 4, 8 and 9 (except for one strain of race 3, HRI8819) and absent or highly divergent in strains of the remaining *Xcc* races. This gene is predicted to encode an oxidoreductase protein and did not show similarity to any previously described avirulence protein in the RefSeq database or T3Es of *Xanthomonas* spp. listed in the *Xanthomonas* Resource database (http://www.xanthomonas.org/t3e.html). In the gene-for-gene model, *A1c* is predicted to confer avirulence in *B. carinata* line PIC1 and *B. juncea* line FBLM2 (a line derived from *B. juncea* cv. FBLM) (Table 2, Chapter 1). Castaneda *et al.* (2005) showed that the *xopAH* gene (syn. *avrXccFM*, *avrXccC*) confers avirulence to a strain of race 3 (ATCC33913$^{T}$) in *B. juncea* cv. FBLM. The *xopAH* gene of strain LMG8004 (race 9) was also shown to confer avirulence in a different *B. juncea* genotype (cv. Guangtou) (He *et al.*, 2007). These strains were compared in this study and have the *A1c* candidate gene. However, the predicted product of the *A1c* candidate gene did not show similarity to XopAH sequences from *X. campestris* strains in BLASTX searches. It is therefore possible

that more than one gene contributes to avirulence of strains of *Xcc* races in *B. carinata* line PIC1 and *B. juncea* line FBLM2.

In present study, genome-wide searches for candidate avirulence genes were performed based on the assumption that genes present/absent (or highly divergence in sequence) across strains of different races, are candidate determinants of avirulence in specific host differentials. However, differences in avirulence between races may also be determined by subtle allelic variation of genes present across all races, and such genes could not be detected by the method used in this study. Nevertheless, both scenarios (presence/absence or subtle allelic variation in avirulence genes) are possible as it was reported for avirulence determinants of races of *Pseudomonas syringae* pv. *phaseolicola* (Stevens *et al*., 1998).

In summary, the present study provides a tractable list of genes for future studies, which may contain key determinants of the distinct modes of pathogenesis of *Xcr* and *Xcc* and host range of races within each of these pathovars.

# CHAPTER 6. General discussion

*Arabidopsis thaliana* has proved to be an excellent plant model for studying the molecular mechanisms underlying plant-pathogen interactions, supported by extensive genetic resources and tools made available to the research community (Nishimura & Dangl, 2010). The study of the closely related pathogens *Xanthomonas campestris* pv. *raphani* (*Xcr*) and *X. campestris* pv. *campestris* (*Xcc*) in this well-established plant model may contribute to a better understanding of the molecular mechanisms underlying distinct modes of pathogenesis and associated disease resistance mechanisms. This provides an important bridge to meet challenges in future food production. Both pathovars infect common *Brassicaceae* hosts, but cause distinct diseases. *Xcr* strains colonize preferentially the parenchyma tissues and cause a leaf spot disease (White, 1930; Tamura *et al.*, 1994; Vicente *et al.*, 2006) whereas *Xcc* strains invade primarily the vascular system of the host and cause black rot disease (Alvarez, 2000). Strains of both pathovars have been reported to infect *A. thaliana* but most literature has been dedicated to explore the *Xcc-A. thaliana* pathosystem (Buell, 2002).

The major aim of this PhD research was to explore the interactions between *X. campestris* and *A. thaliana*, with a particular focus on *Xcr* and to study the genetic basis of disease resistance. Previous phenotypic screens did not identify clear differential responses among *A. thaliana* accessions to specific *Xcr* strains (Davis *et al.*, 1991; Parker *et al.*, 1993), which may have been due to the inoculation method used as infiltration of leaves with bacterial suspensions bypasses possible defence mechanisms associated with the stomata entry of this pathogen. The selection of strains and/or *A. thaliana* accessions tested could also have limited the results obtained. However, as shown in the current study, *Xcr* strains can cause reproducible leaf spot symptoms in spray-inoculated *A. thaliana* plants, similar to those caused in natural hosts (White, 1930; Tamura *et al.*, 1994; Vicente *et al.*, 2006; Fargier & Manceau, 2007). The spray inoculation method also allowed rapid screening of multiple plants, which constitutes a major advantage for genetic studies. In contrast, the wound inoculation method tested in inoculations with *Xcr* strains

(which is generally used in inoculations of *A. thaliana* with *Xcc* strains; Meyer *et al*., 2005), was laborious and produced more variable results in comparison to the results obtained with the spray inoculation method.

Phenotypic variation was identified among diverse *A. thaliana* accessions in response to representative strains of each of the three known *Xcr* races (Vicente *et al.*, 2006), including the 19 parents of a mapping inbred population that was proven to be an excellent tool for mapping loci associated to resistance to *Xcr* in this study (MAGIC lines; Kover *et al.*, 2009). Sources of broad resistance and broad susceptibility to multiple strains of *Xcr* as well as accessions that discriminate between multiple strains of each of the three *Xcr* races were also identified (as summarized in Table 35). The interactions were similar to the phenotypic variation described previously between *Xcr* races and host differential lines of *Brassica* spp. and radish (Vicente *et al.*, 2006). However, sources of resistance to all three races have not yet been identified in other *Brassicaceae* species. Thus, the broad-spectrum resistance observed in *A. thaliana* became a primary focus of the current study. Altogether, these findings support the usefulness of this pathosystem to study the genetic basis of disease resistance to strains of *Xcr*.

The summary of predicted gene-for-gene interactions between selected *A. thaliana* accessions and *Xcr* races shown in Table 35 is based on the following lines of evidence from this thesis research. Interval mapping using *A. thaliana* MAGIC lines allowed the identification of two major effect loci that control resistance to a strain of *Xcr* race 2 (HRI8305), including one on the bottom arm of chromosome 3 and one on the bottom arm of chromosome 5 (designated *RXCR1* and *RXCR2,* respectively). *RXCR1* was confirmed as a single gene conferring resistance in Columbia (gene accession At3g57710) by fine-mapping and verification by loss- and gain-of-function experiments. *RXCR2* was mapped to a 6.27 Mb interval with Oy-0 as a representative accession that contains this locus. Oy-0 is resistant to strains of *Xcr* races 1 and 2 (but not race 3) and it was predicted to contain a non-functional *RXCR1* allele for resistance to *Xcr* race 2 based on the observation of susceptible progeny derived from a cross between the resistant parents Col-0 and Oy-0. Further mapping using a two-parent recombinant inbred line (RIL) population (Nd-1×Oy-0) confirmed *RXCR2* as a major effect resistance locus in Oy-0 to *Xcr* race 2 (Sonawala, 2013) and it is assumed in the gene-for-gene model as a single resistance

gene. And finally, a third *R*-gene is proposed to explain the broad-spectrum resistance of Col-0 to all three *Xcr* races, because the *RXCR1* Col-0 allele is required for resistance to strains of *Xcr* races 2 and 3, but is insufficient to explain resistance to *Xcr* race 1. This is based on phenotyping of a Col-0 *rxcr1*-knockout mutant and a $F_9$ Col-0×Nd-1 inbred line predicted to have the *RXCR1* susceptible allele from Nd-1, which were both susceptible to strains of *Xcr* races 2 and 3, but not to a strain of *Xcr* race 1. Assuming a minimum number of genes to explain the interactions between other *A. thaliana* accessions and *Xcr* races presented in Table 35, the *R3* gene in Col-0 is also postulated in the accessions Po-0 and Rsch-4 which are resistant to *Xcr* race 1, but susceptible to *Xcr* races 2 and 3.

**Table 35**. Summary of postulated gene-for-gene interactions between selected *Arabidopsis thaliana* accessions and races of *Xanthomonas campestris* pv. *raphani*

| | | | | ***Xcr* races and predicted avirulence genes (*avr*)** | | |
|---|---|---|---|---|---|---|
| | | | | **1** | **2** | **3** |
| | | | | *avr3* | *avrRxcr1* | *avrRxcr1* |
| ***A. thaliana* accessions** | **Resistance genes** | | | *avrRxcr2?* | *avrRxcr2* | |
| **Broad resistance** | | | | | | |
| Col-0 | *RXCR1** | | *R3* | − | − | − |
| Wil-2 | *RXCR1* | *RXCR2* | *R3?* | − | − | − |
| **Broad susceptibility** | | | | | | |
| Nd-1, Zu-0 | | | | + | + | + |
| **Differential interactions** | | | | | | |
| Oy-0 | | *RXCR2* | *R3?* | − | − | + |
| Po-0, Rsch-4 | | | *R3* | − | + | + |

Symbols and abbreviation: +, compatible interaction (susceptibility); −, incompatible interaction (resistance); *X. campestris* pv. *raphani* (*Xcr*).

* Gene confirmed to confer resistance in *A. thaliana* Col-0 to strains of *X. campestris* pv. *raphani* races 2 and 3, based on results from the present study and results presented by Huard-Chauveau *et al.* (2013).

The accession Wil-2 has been added to the genetic model as an example that may contain functional resistance alleles of both *RXCR1* and *RXCR2*. This is based on the analysis of publicly available genome-sequencing data, which indicates that this accession contains the same allele of *RXCR1* as in Col-0 for resistance to *Xcr* race 2 (Table 19); and in the prediction from the mapping analysis (using MAGIC lines) that Wil-2 shares a resistance allele on chromosome 5 with Oy-0 (Figure 10b). Whether *RXCR2* also provides resistance to *Xcr* race 1 is unknown and therefore the

*R3* gene is proposed as an alternative explanation for resistance in Wil-2 and Oy-0 to this race.

For future research, molecular identification of *RXCR2* and the postulated *R3* gene conferring resistance to *Xcr* race 1 will be important targets to verify in the proposed genetic model. Fine-mapping of *RXCR2* locus and identification of candidate genes could be pursued by developing more molecular markers in the Nd-1×Oy-0 RIL population. Mapping loci controlling resistance to *Xcr* race 1 could be achieved using the *A. thaliana* MAGIC population and it would confirm whether resistance to this race is also mapped to the *RXCR2* locus and/or other loci. Fine-mapping of resistance loci and identification of candidate gene(s) could be achieved using the Col×Nd-1 RIL population that was used in this study for fine-mapping of *RXCR1*. The MAGIC population could also be used to identify other potential loci than *RXCR1*, for resistance to *Xcr* race 3.

Importantly, phenotypic variation in responses to *Xcc* strains was also identified among the diverse *A. thaliana* accessions tested with *Xcr* strains including the parents of the MAGIC population. Accessions showing clear differential responses to representative strains of the most common *Xcc* races (1 and 4) and the broad virulent *Xcc* race 6 were identified. Overall, these phenotypic screenings provide useful information for future studies aiming to identify the molecular mechanisms underlying resistance to *X. campestris*. For example, Wil-2 exhibited resistance to all strains of *Xcr* and *Xcc* tested whereas Col-0 was found resistant to all *Xcr* and *Xcc* strains tested except *Xcc* race 6. This broad-spectrum resistance to both pathovars is most likely explained by a combination of multiple *R*-genes including *RXCR1*.

*RXCR1*-mediated disease resistance in Col-0 is not pathovar specific. This gene was also identified in an independent study that investigated the genetic basis of resistance in *A. thaliana* to a *Xcc* strain of race 3 (ATCC33913) (Huard-Chauveau *et al.*, 2013). In that study, the Col-0 allele of *RXCR1* (or *RKS1* as designated by the authors) was also reported to confer resistance to strains of other *Xcc* races (1, 5, 7 and 9), as well as strains of the *X. campestris* pathovars *incanae*, *armoraciae* and *raphani*. *RXCR1*/*RKS1*-mediated resistance to *Xcr* was confirmed in that study using two strains of race 3 and these results together with the results of the present study confirm that this gene confers resistance to strains of at least two races of *Xcr* (races

2 and 3). *RXCR1*/*RKS1* encodes a kinase-like protein that was recently described to form a complex with an NB-LRR protein (ZAR1) for recognition of the type III effector AvrAC (or XopAC) from *Xcc* strain LMG8004, when this effector uridylates a receptor-like cytoplasmic kinase PBL2 (Wang *et al.*, 2015). *RXCR1*-mediated resistance to *Xcr* strains may also involve recognition of the same effector because the presence of the *xopAC* gene has been reported in the two published genome sequences of *Xcr* strains (Bogdanove *et al.*, 2011; Roux *et al.*, 2015) and it was also confirmed in all *Xcr* genomes sequenced in the present study (data not shown). Future work could confirm whether *RXCR1*-mediated resistance to *Xcr* is also mediated by recognition of this effector.

To date, genes conferring resistance to either *Xcr* or *Xcc* have not yet been cloned in *Brassicaceae* crop species (Vicente & Holub, 2013). However, *RXCR1*/*RKS1* orthologous genes have been identified in the genome sequence of *Brassica rapa* (Huard-Chauveau et al., 2013) and *B. oleracea* (Parkin et al., 2014). Also, ZAR1 has been reported to be one of the few NB-LRR genes conserved across *A. thaliana* and four other *Brassicaceae* species analysed to date (namely *B. rapa*, *Arabidopsis lyrata*, *Capsella rubella*, and *Eutrema salsugineum*) (Peele *et al.*, 2014). Whether orthologues of *RXCR1* and *ZAR1* also confer resistance to *Xcr* and *Xcc* strains in *Brassicaceae* crop species will be an important question to address in the future for improving resistance to *X. campestris* in crops. Other genes conferring resistance to *Xcc* strains have been recently identified in *A. thaliana* (Debieu *et al.*, 2016). One gene encoding a protein of unknown function was reported to confer resistance in the *A. thaliana* accession Col-0 to a strain of *Xcc* race 2 and the well-known resistance gene pair *RPS4*-*RRS1* was also reported to confer resistance to a strain of *Xcc* race 6 in the *A. thaliana* accession Ws-0. However, no evidence was found that these genes are involved in resistance to *Xcr* (Debieu *et al.*, 2016), and *RXCR1* remains the only gene identified to date involved in resistance to strains of this pathovar.

On the bacteria side, a genome-wide approach was developed with bioinformatics support from D. Studholme and J. Harrison (University of Exeter, UK) to identify candidate determinants of the distinct pathogenic features of *Xcr* and *Xcc* as well as avirulence determinants of races within these pathovars. For this purpose, whole-genome sequencing data from a diverse collection of *Xcr* and *Xcc*

strains including representatives of all races defined within each pathovar was used. This is the first study that compares whole-genome sequencing data from multiple *Xcr* and *Xcc* strains including representatives of each race defined within each pathovar. Whole-genome sequencing data from 32 *X. campestris* strains was used to create a reference pan-genome which allowed to perform specific searches for genes differentially present/absent (or highly divergent in sequence) between different groups of strains.

Genes differentially present/absent (or highly divergent in sequence) between strains of *Xcr* and *Xcc* were identified providing a list of candidate determinants of their distinct modes of pathogenesis. Functional studies of these genes may provide new insights towards a better understanding of the non-vascular and vascular behaviour of *Xcr* and *Xcc*, respectively. Five of these genes encode predicted or known type III effectors and three have been previously described to have an effect in *Xcc* virulence, namely *avrBs2*, *xopAM* and *xopQ* (Xu *et al.*, 2006; Jiang *et al.*, 2009). In particular, the genes identified in *Xcr* but not in *Xcc*, have not yet been studied and it would be interesting to further investigate whether they have a role in pathogenicity. Candidate avirulence genes shared by strains of specific races within each of these pathovars as predicted in the published gene-for-gene models to explain the interactions with *Brassicaceae* host differentials (*Brassica* spp. and radish) (Vicente *et al.*, 2006; Vicente & Holub, 2013), were also identified. None of these genes have been previously reported to have an avirulence function in *Xcr* or *Xcc* and could be investigated in future studies to determine whether they confer avirulence to races of these pathovars in *Brassicaceae* host differentials. Although searches were only performed for avirulence genes predicted in those gene-for-gene models, the two candidate avirulence genes identified in *Xcr* race 1 (but not in *Xcr* races 2 and 3) are also candidates for the *avr3* gene predicted in the gene-for-gene model proposed in this study for *Xcr-A. thaliana* interactions (Table 35).

And finally, I had the opportunity to investigate bacteria causing a direct impact in food production in Mauritius. This involved molecular and pathogenic characterization of bacteria associated with severe outbreaks of a leaf spot and blight disease occurring in vegetable brassica crops in that country. *X. campestris* strains were isolated from diseased leaves provided by the field pathologist R. Lobin in 2012. These strains and strains isolated previously in 2009, caused vascular

infections similar to those caused by reference *Xcc* strains used for comparison in pathogenicity tests. However, the strains from Mauritius showed a greater capability to cause leaf blight symptoms than the reference strains. The development of small leaf spots in some plants was also observed, but they were clearly distinct from leaf spots caused by *Xcr*. The strains from Mauritius were confirmed to be different from *X. campestris* strains associated with similar outbreaks of a leaf spot and blight disease of brassicas reported in South Carolina (USA) (Wechter *et al.*, 2008). Strains from Mauritius were identified as *Xcc* race 4 whereas strains from South Carolina were identified as *Xcc* race 6, based on their interactions with *Brassicaceae* host differentials. Strains from both regions also fell in separate clades in phylogenetic trees based on multiple gene sequences (Figures 30 and 31). Interestingly, pathogenic *Pseudomonas* sp. strains were also isolated from diseased leaves received from Mauritius and it is possible that the disease phenotype observed in the field is a result of co-infection of plants by *Xanthomonas* and *Pseudomonas* pathogens as previously reported by Zhao *et al*. (2000a, 2000b) for a leaf spot disease of brassicas grown in Oklahoma (USA).

In conclusion, the *A. thaliana*-*Xcr* pathosystem is an excellent system to study plant-pathogen interactions. In contrast to the laborious wound inoculation method required to study the interactions between *A. thaliana* and the vascular pathogen *Xcc*, the spray inoculation method used for *Xcr* allows screening of multiple plants in a short period of time, which is an important advantage for genetic studies. Over the course of mapping the *RXCR1* locus, more than 2400 plants were phenotyped which was strongly facilitated by the inoculation method used. Undoubtedly the resources and tools available to perform genetic studies using *A. thaliana* also facilitated the identification of *RXCR1* gene in a relatively short period of time. Although yet unexplored, this is a suitable pathosystem to study the molecular basis of plant-pathogen interactions and from my experience I encourage the continuation of research on this pathosystem.

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# APPENDIX 1. List of validly named *Xanthomonas* species

A complete list of all validly named *Xanthomonas* species listed in the LPSN website (List of Prokaryotic names with Standing in Nomenclature; http://www.bacterio.net; Parte, 2014) at the time of this research (August, 2015), is presented together with some of the respective type strain accessions, origin of isolation and the number of validly published pathovars within each species as listed by Bull *et al.* (2010a; 2012).

| Species name | Type strain | Isolation [a] | | | Number of pathovars |
|---|---|---|---|---|---|
| | | Plant host | Country | Year | |
| ***Xanthomonas albilineans*** (Ashby 1929) Dowson 1943 | CFBP2523 ICMP196 LMG494 NCPPB2969 | *Saccharum officinarum* | Fiji | 1961 | 0 |
| ***X. alfalfae*** (ex Riker *et al.* 1935) Schaad *et al.* 2007 | LMG495 NCPPB4412 | *Medicago sativa* | India | na | 0 |
| ***X. arboricola*** Vauterin *et al.* 1995 | CFBP2528 LMG747 NCPPB411 | *Juglans regia* | New Zealand | 1956 | 6 |
| ***X. axonopodis*** Starr and Garces 1950 | CFBP4924 ICMP50 LMG538 NCPPB457 | *Axonopus scoparius* | Colombia | 1949 | 44 |
| ***X. bromi*** Vauterin *et al.* 1995 | CFBP1976 ICMP12545 LMG947 NCPPB4343 | *Bromus carinatus* | France | 1980 | 0 |
| ***X. campestris*** (Pammel 1895) Dowson 1939 [T] | ATCC33913 CFBP5241 HRI5212 NCPPB528 | *Brassica oleracea* var. *gemmifera* | UK | 1957 | 7 |
| ***X. cassavae*** (ex Wiehe and Dowson 1953) Vauterin *et al.* 1995 | CFBP4642 ICMP204 NCPPB101 | *Manihot esculenta* | Malawi | 1951 | 0 |
| ***X. citri*** (ex Hasse 1915) Gabriel *et al.* 1989 | LMG9322 | *Citrus aurantifolia* | USA | 1989 | 0 |
| ***X. codiaei*** Vauterin *et al.* 1995 | CFBP4690 ICMP9513 LMG8678 NCPPB4350 | *Codiacum variegatum* | USA | 1987 | 0 |
| ***X. cucurbitae*** (ex Bryan 1926) Vauterin *et al.* 1995 | CFBP2542 ICMP2299 LMG690 NCPPB2597 | *Cucurbita maxima* | New Zealand | 1968 | 0 |
| ***X. cynarae*** Trébaol *et al.* 2000 | CFBP4188 ICMP16775 NCPPB4356 | *Cynara scolymus* | France | 1996 | 0 |
| ***X. dyei*** Young *et al.* 2010 | CFBP7245 DSM19128 ICMP12167 NCPPB4446 | *Metrosideros excels* | New Zealand | na | 3 |
| ***X. euvesicatoria*** Jones *et al.* 2006 | CFBP6864 DSM19128 ICMP109 NCPPB2968 | *Capsicum frutescens* | USA | 1947 | 0 |
| ***X. fragariae*** Kennedy and King 1962 | CFBP2157 ICMP5715 LMG708 NCPPB1469 | *Fragaria* sp. | USA | 1960 | 0 |

*Continues next page*

| Species name | Type strain | Isolation [a] | | | Number of pathovars |
|---|---|---|---|---|---|
| | | Plant host | Country | Year | |
| ***X. fuscans*** Schaad *et al.* 2007 | CFBP6165<br>LMG826<br>NCPPB381 | *Phaseolus vulgaris* | Canada | 1957 | 0 |
| ***X. gardneri*** (ex Šutič 1957) Jones *et al.* 2006 | ATCC19865<br>DSM19127<br>ICMP16689 | *Lycopersicon esculentum* | Yugoslavia | na | 0 |
| ***X. hortorum*** Vauterin *et al.* 1995 | ICMP453<br>LMG733<br>NCPPB939 | *Hedera helix* | USA | 1943 | 0 |
| ***X. hyacinthi*** (ex Wakker 1883) Vauterin *et al.* 1995 | CFBP1156<br>ICMP189<br>LMG739 | *Hyacinthus orientalis* | Netherlands | 1958 | 0 |
| ***X. melonis*** Vauterin *et al.* 1995 | ICMP8682<br>LMG8670<br>NCPPB3434 | *Cucumis melo* | Brazil | na | 0 |
| ***X. oryzae*** (ex Ishiyama 1922) Swings *et al.* 1990 | CFBP2532<br>ICMP3125<br>LMG5047 | *Oryza sativa* | India | 1965 | 2 |
| ***X. perforans*** Jones *et al.* 2006 | CFBP7293<br>DSM18975<br>ICMP16690<br>NCPPB4321 | *Lycopersicon esculentum* | USA | 1991 | 0 |
| ***X. pisi*** (ex Goto and Okabe 1958) Vauterin *et al.* 1995 | CFBP4643<br>ICMP570<br>LMG847 | *Pisum sativum* | Japan | 1957 | 0 |
| ***X. populi*** (ex Ridé 1958) van der Mooter and Swings 1990 | CFBP1817<br>ICMP5816<br>LMG5743 | *Populus canadensis* cv. *regenerata* | France | 1957 | 0 |
| ***X. sacchari*** Vauterin *et al.* 1995 | CFBP4641<br>ICMP16916<br>LMG471 | *Saccharum officinarum* | France | na | 0 |
| ***X. theicola*** (Uehara *et al.* 1980) Vauterin *et al.* 1995 | CFBP4691<br>ICMP6774<br>LMG8684 | Camellia sinensis | Japan | 1974 | 0 |
| ***X. translucens*** (*ex* Jones *et al.* 1917) Vauterin *et al.* 1995 | NCPPB973<br>ICMP5752<br>LMG876 | *Hordeum vulgare* | USA | | 10 |
| ***X. vasicola*** Vauterin *et al.* 1995 | CFBP2543<br>ICMP3103<br>LMG736 | *Sorghum vulgare* | New Zealand | 1969 | 0 |
| ***X. vesicatoria*** (ex Doidge 1920) Vauterin *et al.* 1995 | ATCC35937<br>CFBP2537<br>HRI5235<br>ICMP63<br>NCPPB422 | *Lycopersicon esculentum* | New Zealand | 1955 | 0 |

Abbreviations: ATCC, American Type Culture Collection, Manassas, USA; CFBP, French Collection of Plant associated Bacteria, INRA, Angers, France; HRI, formerly Horticulture Research International, now University of Warwick Crop Centre, Wellesbourne, UK; ICMP, International Collection of Microorganisms from Plants, Lincoln, New Zealand; LMG, Laboratory for Microbiology, Faculty of Sciences, Ghent University, Ghent, Belgium; na, not available; NCPPB, National Collection of Plant Pathogenic Bacteria, Fera, York, UK.

[a] Information regarding the origin of isolation of each type strain was retrieved from the online catalogues of the culture collections indicated in the type strain accessions as follows: ATCC (http://www.lgcstandards-atcc.org); CFBP (http://www-intranet.angers.inra.fr/cfbp); ICMP (http://www.landcareresearch.co.nz); LMG (http://bccm.belspo.be); NCPPB (http://ncppb.fera.defra.gov.uk).

# APPENDIX 2. Culture media

## 1. King's B agar (King *et al.*, 1954)

Proteose peptone .....20.0 g
$K_2HPO_4$ (potassium dihydrogen phosphate) .....1.5 g
$MgSO_4.7H_2O$ (magnesium sulfate 7-hydrate) .....1.5 g
Agar .....15.0 g
Glycerol ..... 10 ml or 12.6 g
Distilled water ..... 1000 ml

Dissolve the glycerol in water and then suspend the remaining components of the medium. Autoclave at 120 °C and approximately 17.5 psi for 15 min. Allow the medium to cool down to approximately 50 °C and poor the medium into to plates.

## 2. Yeast dextrose calcium carbonate agar (Schaad *et al.*, 2001)

Yeast extract .....10.0 g
Dextrose (glucose) .....20.0 g
Calcium carbonate (finely grounded) .....20.0 g
Agar .....15.0 g
Distilled water ..... 1000 ml

Autoclave at 120 °C and approximately 17.5 psi for 15 min. Allow the medium to cool down to approximately 50 °C and poor the medium into to plates.

## 3. Luria-Bertani (LB) medium (Bent, 2006)

Bacto tryptone .....10 g
Yeast extract .....5 g
NaCl .....5 g
Deionized water ..... 1000 ml

Add 15 g agar if preparing solid media. Autoclave at 120 °C and approximately 17.5 psi for 15 min. The addition of antibiotics should be performed after the media has cooled down below 65 °C.

## APPENDIX 3. Determination of *Xanthomonas campestris* inoculum cell number

The absorbance (A) of bacterial suspensions that corresponds to a concentration of $10^8$ bacterial cells per ml was determined and used as a reference for preparation of suspensions used in plant inoculations carried out in the present study. The method used was adapted from methods previously described by Király *et al.* (1974) and Vicente (2000). The *Xanthomonas campestris* pv. *raphani* strain HRI8305 was used for this purpose.

The bacterial strain HRI8305 was streaked on King's B medium (King *et al.*, 1954; Appendix 2) and incubated at 28 °C for 48 h. Six bacterial suspensions were prepared in sterile distilled water and the absorbance was measured and adjusted to 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5. All measurements were carried out at 640 nm using a spectrophotometer (Spectronic 21, Milton Roy). Serial 10-fold dilutions were prepared from each suspension up to the $10^{-7}$ dilution. Aliquots of 100 µl of $10^{-5}$, $10^{-6}$ and $10^{-}7$ dilutions were spread on King's B medium plates using sterile disposable L-shaped spreaders. Three replicate plates per dilution were prepared and plates were incubated at 28 °C for 24 to 48 h.

Colony-forming units (cfu) were counted in plates with approximately 30 to 300 cfu. The bacterial cell concentration of each initial suspension was then estimated based on the average number of colonies counted. The results obtained are presented in Table 3a. The $A_{640nm}$ 0.2 corresponded to a cell concentration of approximately $2\times10^8$ cfu/ml and it was selected as a reference for the inoculum preparations carried out in the present study.

**Table 3a**. Absorbance of bacterial suspensions measured at 640 nm and respective average number of cells per ml (cfu/ml)

| Suspension | Absorbance | Number of living cells (cfu/ml) |
|---|---|---|
| 1 | 0.05 | $2.4\times10^7$ |
| 2 | 0.1 | $9.0\times10^7$ |
| 3 | 0.2 | $2.1\times10^8$ |
| 4 | 0.3 | $3.9\times10^8$ |
| 5 | 0.4 | $6.7\times10^8$ |
| 6 | 0.5 | $7.4\times10^8$ |

## APPENDIX 4. *Arabidopsis thaliana* MAGIC inbred lines used in this study and respective interaction phenotypes with *Xanthomonas campestris* pv. *raphani* strain HRI8305

The maximum phenotype score observed in two plants of each of the 353 MAGIC inbred lines spray-inoculated with strain HRI8305, are presented. The phenotypes were assessed 10 days after inoculation using a six-point scale developed in this study (Figure 4, Chapter 2).

| MAGIC line | Phenotype score | MAGIC line | Phenotype score | MAGIC line | Phenotype score |
|---|---|---|---|---|---|
| 5 | 1 | 54 | 4 | 101 | 4 |
| 6 | 0 | 55 | 2 | 103 | 3 |
| 7 | 1 | 57 | 0 | 104 | 0 |
| 9 | 2 | 59 | 1 | 105 | 0 |
| 10 | 0 | 60 | 2 | 107 | 3 |
| 11 | 1 | 61 | 2 | 109 | 0 |
| 12 | 0 | 62 | 1 | 111 | 3 |
| 13 | 2 | 63 | 3 | 112 | 2 |
| 14 | 1 | 64 | 3 | 113 | 2 |
| 16 | 1 | 66 | 0 | 114 | 2 |
| 17 | 2 | 67 | 2 | 115 | 2 |
| 18 | 4 | 68 | 0 | 116 | 0 |
| 20 | 0 | 70 | 0 | 117 | 1 |
| 21 | 3 | 71 | 2 | 119 | 1 |
| 23 | 2 | 72 | 4 | 121 | 3 |
| 24 | 3 | 73 | 0 | 122 | 1 |
| 25 | 3 | 75 | 2 | 123 | 2 |
| 26 | 1 | 76 | 2 | 124 | 3 |
| 27 | 3 | 77 | 3 | 125 | 3 |
| 28 | 0 | 79 | 1 | 126 | 0 |
| 30 | 0 | 80 | 2 | 127 | 1 |
| 31 | 1 | 81 | 1 | 128 | 2 |
| 32 | 3 | 83 | 0 | 129 | 1 |
| 34 | 0 | 84 | 2 | 130 | 2 |
| 35 | 2 | 85 | 3 | 131 | 0 |
| 37 | 3 | 86 | 2 | 133 | 3 |
| 38 | 3 | 88 | 0 | 135 | 0 |
| 39 | 2 | 89 | 3 | 138 | 2 |
| 40 | 2 | 90 | 0 | 152 | 3 |
| 42 | 0 | 91 | 1 | 153 | 2 |
| 44 | 1 | 92 | 3 | 156 | 1 |
| 45 | 3 | 93 | 0 | 158 | 1 |
| 46 | 0 | 94 | 0 | 159 | 0 |
| 49 | 0 | 95 | 2 | 160 | 0 |
| 50 | 1 | 96 | 3 | 161 | 0 |
| 51 | 1 | 97 | 3 | 162 | 0 |
| 52 | 0 | 98 | 0 | 163 | 0 |
| 53 | 0 | 99 | 4 | 164 | 0 |

*Continues next page*

APPENDIX 4 (continued)

| MAGIC line | Phenotype score |
|---|---|
| 165 | 3 |
| 166 | 2 |
| 167 | 0 |
| 168 | 0 |
| 170 | 0 |
| 171 | 0 |
| 174 | 3 |
| 175 | 0 |
| 176 | 1 |
| 177 | 0 |
| 178 | 3 |
| 180 | 0 |
| 181 | 0 |
| 182 | 2 |
| 183 | 1 |
| 185 | 0 |
| 187 | 2 |
| 188 | 0 |
| 189 | 0 |
| 190 | 1 |
| 191 | 1 |
| 192 | 3 |
| 193 | 0 |
| 195 | 0 |
| 196 | 0 |
| 197 | 0 |
| 199 | 2 |
| 200 | 0 |
| 203 | 1 |
| 205 | 2 |
| 207 | 0 |
| 209 | 3 |
| 265 | 1 |
| 276 | 2 |
| 278 | 0 |
| 280 | 0 |
| 282 | 3 |
| 283 | 3 |
| 284 | 0 |
| 285 | 3 |
| 286 | 3 |
| 287 | 4 |
| 289 | 0 |
| 290 | 2 |
| 291 | 1 |
| 292 | 0 |
| 293 | 2 |
| 295 | 1 |
| 296 | 1 |
| 298 | 3 |
| 299 | 5 |
| 302 | 4 |
| 303 | 3 |
| 304 | 2 |
| 305 | 5 |
| 307 | 5 |
| 308 | 0 |
| 309 | 1 |
| 310 | 1 |
| 311 | 0 |
| 312 | 5 |
| 299 | 5 |
| 302 | 4 |
| 303 | 3 |
| 304 | 2 |
| 305 | 5 |
| 307 | 5 |
| 308 | 0 |
| 309 | 1 |
| 310 | 1 |
| 311 | 0 |
| 312 | 5 |
| 313 | 1 |
| 314 | 0 |
| 315 | 3 |
| 316 | 2 |
| 317 | 3 |
| 318 | 0 |
| 319 | 3 |
| 320 | 1 |
| 321 | 3 |
| 322 | 1 |
| 323 | 0 |
| 324 | 1 |
| 325 | 1 |
| 326 | 3 |
| 327 | 3 |
| 328 | 3 |
| 329 | 3 |
| 330 | 3 |
| 331 | 1 |
| 332 | 0 |
| 333 | 4 |
| 334 | 0 |
| 336 | 3 |
| 337 | 1 |
| 338 | 4 |
| 339 | 2 |
| 340 | 3 |
| 342 | 3 |
| 344 | 2 |
| 345 | 4 |
| 346 | 0 |
| 347 | 0 |
| 348 | 5 |
| 349 | 5 |
| 351 | 2 |
| 352 | 1 |
| 353 | 3 |
| 354 | 0 |
| 355 | 0 |
| 358 | 0 |
| 360 | 5 |
| 362 | 3 |
| 363 | 3 |
| 364 | 3 |
| 365 | 3 |
| 366 | 0 |
| 367 | 3 |
| 368 | 4 |
| 369 | 4 |
| 370 | 3 |
| 371 | 1 |
| 372 | 5 |
| 373 | 4 |
| 375 | 5 |
| 376 | 4 |
| 377 | 0 |
| 378 | 1 |
| 379 | 1 |
| 380 | 2 |
| 381 | 4 |
| 370 | 3 |
| 371 | 1 |
| 372 | 5 |
| 373 | 4 |
| 375 | 5 |
| 376 | 4 |
| 377 | 0 |
| 378 | 1 |
| 379 | 1 |
| 380 | 2 |
| 381 | 4 |
| 382 | 4 |
| 385 | 3 |
| 386 | 4 |
| 388 | 2 |
| 389 | 3 |
| 390 | 3 |
| 391 | 3 |
| 392 | 4 |
| 393 | 3 |
| 395 | 4 |
| 396 | 3 |
| 397 | 2 |
| 398 | 4 |
| 400 | 1 |
| 401 | 0 |
| 402 | 2 |
| 403 | 2 |
| 407 | 0 |
| 409 | 4 |
| 413 | 0 |
| 414 | 5 |
| 415 | 1 |
| 416 | 4 |
| 418 | 5 |
| 419 | 4 |
| 420 | 0 |
| 421 | 5 |
| 423 | 4 |

*Continues next page*

APPENDIX 4 (continued)

| MAGIC line | Phenotype score |
|---|---|
| 424 | 3 |
| 425 | 1 |
| 426 | 5 |
| 427 | 2 |
| 428 | 0 |
| 429 | 0 |
| 430 | 5 |
| 431 | 4 |
| 432 | 3 |
| 433 | 1 |
| 434 | 1 |
| 435 | 4 |
| 436 | 4 |
| 437 | 2 |
| 438 | 1 |
| 440 | 2 |
| 441 | 0 |
| 442 | 1 |
| 443 | 1 |
| 446 | 3 |
| 447 | 3 |
| 448 | 3 |
| 449 | 4 |
| 450 | 2 |
| 451 | 3 |
| 453 | 3 |
| 454 | 0 |
| 455 | 4 |
| 456 | 4 |
| 457 | 5 |
| 460 | 2 |

| MAGIC line | Phenotype score |
|---|---|
| 461 | 1 |
| 448 | 3 |
| 449 | 4 |
| 450 | 2 |
| 451 | 3 |
| 453 | 3 |
| 454 | 0 |
| 455 | 4 |
| 456 | 4 |
| 457 | 5 |
| 460 | 2 |
| 461 | 1 |
| 462 | 3 |
| 464 | 3 |
| 465 | 1 |
| 466 | 0 |
| 467 | 0 |
| 468 | 2 |
| 469 | 0 |
| 473 | 5 |
| 474 | 0 |
| 475 | 0 |
| 476 | 4 |
| 477 | 0 |
| 478 | 0 |
| 479 | 0 |
| 480 | 3 |
| 481 | 1 |
| 483 | 0 |
| 484 | 1 |
| 485 | 4 |

| MAGIC line | Phenotype score |
|---|---|
| 487 | 4 |
| 490 | 0 |
| 491 | 1 |
| 492 | 3 |
| 493 | 3 |
| 494 | 0 |
| 495 | 3 |
| 496 | 0 |
| 499 | 3 |
| 501 | 5 |
| 502 | 0 |
| 503 | 0 |
| 504 | 4 |
| 505 | 0 |
| 506 | 3 |
| 507 | 3 |
| 508 | 0 |
| 509 | 4 |
| 510 | 4 |
| 514 | 3 |
| 515 | 0 |
| 516 | 2 |
| 517 | 3 |
| 519 | 3 |
| 520 | 4 |
| 521 | 4 |

## APPENDIX 5. *Arabidopsis thaliana* recombinant inbred lines derived from a cross of accessions Columbia (Col-0 or Col-5) and Niederzenz (Nd-1) and respective genotypes for molecular markers m57530 and m58310 determined in this study

The genotypes determined in this study of 87 Col×Nd-1 $F_9$ recombinant inbred lines for the molecular markers m57530 and m58310 (described in Table 11, Chapter 3), are presented. 'CC' indicates the homozygous genotype for the Columbia allele (highlighted in green), 'NN' indicates the homozygous genotype for Nd-1 allele (highlighted in yellow) and 'CN' indicates the heterozygous genotype (highlighted in grey). The plant lines showing recombination events between the two molecular markers are indicated within black rectangular lines. Plant lines were selected from a mapping population developed by Holub & Beynon (1997).

| Inbred line | m57530 | m58310 |
|---|---|---|
| 3790 | CC | NN |
| 3800 | CC | CC |
| 3819 | CC | CC |
| 3858 | CC | CC |
| 3860 | NN | NN |
| 3887 | NN | NN |
| 3892 | NN | NN |
| 3893 | NN | NN |
| 8002 | CC | CC |
| 8003 | CC | CC |
| 8004 | NN | NN |
| 8005 | CC | CC |
| 8006 | CC | CC |
| 8007 | CN | CN |
| 8009 | NN | NN |
| 8010 | CC | CC |
| 8011a | CN | NN |
| 8011b | CC | NN |
| 8014 | NN | NN |
| 8015 | CC | CC |
| 8016 | NN | NN |
| 8019 | CC | CC |
| 8022 | CC | CC |
| 8024 | CC | CC |
| 8027 | NN | NN |
| 8028 | NN | NN |
| 8029 | CC | CC |
| 8030 | CC | CC |
| 8032 | NN | NN |
| 8033 | NN | NN |

| Inbred line | m57530 | m58310 |
|---|---|---|
| 8034 | CC | CC |
| 8035 | CC | CC |
| 8036 | CC | CC |
| 8037 | CC | CC |
| 8038 | CC | CC |
| 8039 | CC | CC |
| 8040 | CC | CC |
| 8041 | CC | CC |
| 8042 | CC | CC |
| 8043 | CC | CC |
| 8044 | NN | CC |
| 8045 | CC | CC |
| 8047 | CC | CC |
| 8048 | NN | CC |
| 8050 | CC | CC |
| 8051 | CC | CC |
| 8052 | CC | CC |
| 8053 | NN | NN |
| 8054 | NN | NN |
| 8055 | CC | CC |
| 8056 | NN | NN |
| 8057 | NN | NN |
| 8058 | CC | CC |
| 8059 | NN | NN |
| 8060 | NN | NN |
| 8061 | CC | CC |
| 8063 | CC | CC |
| 8063 | CC | CC |
| 8064 | CC | CC |
| 8065 | NN | NN |

| Inbred line | m57530 | m58310 |
|---|---|---|
| 8066 | NN | NN |
| 8067 | CC | CC |
| 8068 | NN | NN |
| 8069 | NN | NN |
| 8070 | NN | NN |
| 8071 | CC | CC |
| 8072 | CC | CC |
| 8073 | NN | NN |
| 8074 | NN | NN |
| 8075 | CC | CC |
| 8077 | CC | CC |
| 8076 | NN | NN |
| 8078 | NN | NN |
| 8079 | NN | NN |
| 8080 | CC | CC |
| 8082 | CC | CN |
| 8085 | NN | NN |
| 8086 | NN | NN |
| 8087 | CC | CC |
| 8088 | CC | CC |
| 8089 | NN | NN |
| 8090 | CC | CC |
| 8091 | NN | NN |
| 8092 | CC | CC |
| 8093 | CC | CC |
| 8094 | NN | NN |
| 8097 | CC | CC |

## APPENDIX 6. *Arabidopsis thaliana* T-DNA insertion mutants (Col-0 background) used in this study and respective interaction phenotypes with the *Xanthomonas campestris* pv. *raphani* strain HRI8305

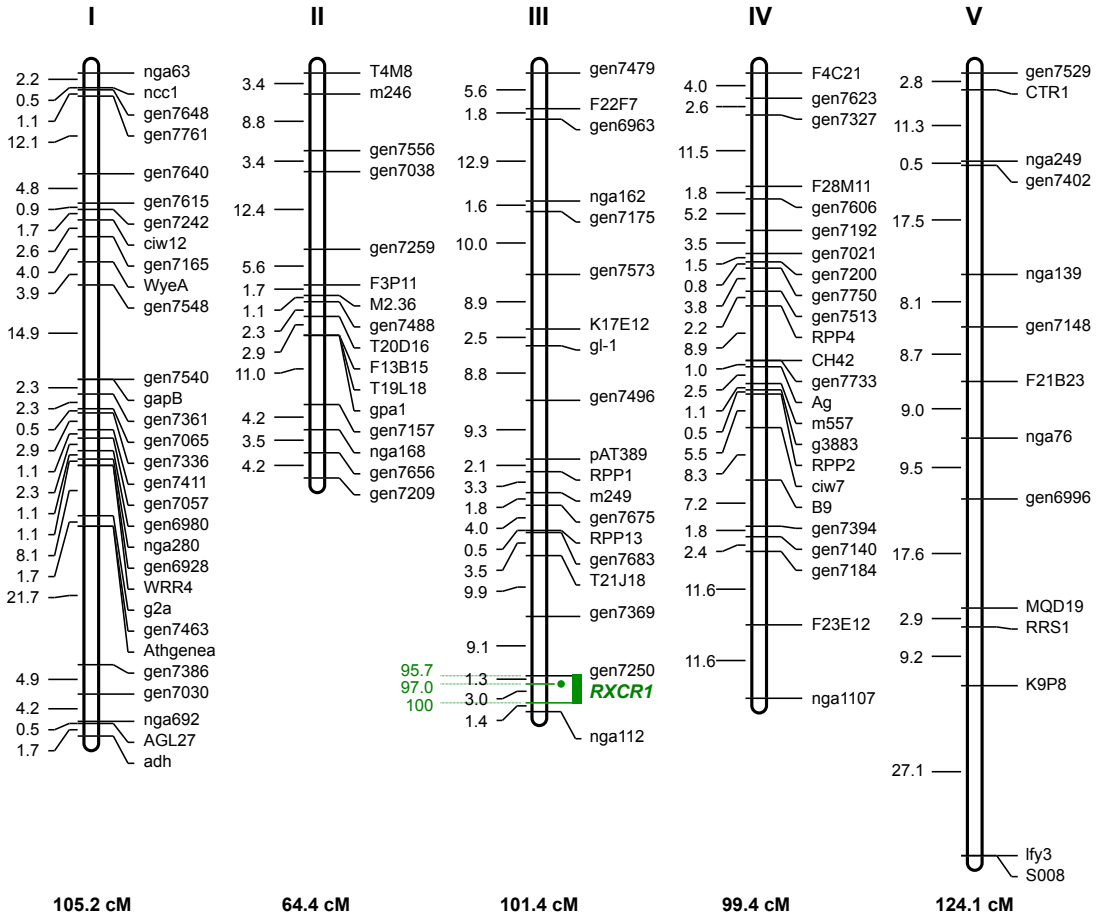
| Gene ID | Mutant line [a] | | Insert site | Segregation status | Interaction phenotype [b] |
|---|---|---|---|---|---|
| | SALK | NASC | | | |
| At3g57660 | SALK116823C | N673273 | UTR3 | Homozygous | 0-1 |
| At3g57700 | SALK145890 | N645890 | Exon | Segregating | 0-1 |
| | SALK025615C | N670449 | UTR5 | Homozygous | 0-2 |
| At3g57710 | SALK204228C | N692905 | Exon | Homozygous | 3-5 |
| At3g57720 | SALK019218C | N684801 | UTR5 | Homozygous | 0-1 |
| At3g57730 | SALK206318C | N695867 | Exon | Homozygous | 0-1 |
| At3g57760 | SALK065826C | N683581 | UTR3 | Homozygous | 0-1 |
| At3g57770 | SALK055617 | N555617 | UTR3 | Segregating | 0-1 |
| | SALK055619 | N555619 | UTR3 | Segregating | 0-1 |
| At3g57800 | SALK063280C | N664650 | Exon | Homozygous | 0-1 |
| | SALK134005C | N669084 | Intron | Homozygous | 0-1 |
| | SALK002003 | N502003 | Exon | Segregating | 0-1 |
| At3g57810 | SALK048627C | N660417 | Promoter | Homozygous | 0-1 |
| | SALK058715C | N660438 | Promoter | Homozygous | 0-1 |
| At3g57820 | SALK025542C | N674177 | Promoter | Homozygous | 0-1 |
| At3g57830 | SALK070935C | N662897 | Exon | Homozygous | 0-1 |
| | SALK061193 | N561193 | Exon | Segregating | 0-1 |
| | SALK022289 | N522289 | Exon | Segregating | 0-1 |
| | SALK058587C | N671459 | Exon | Homozygous | 0-1 |

[a] Mutant lines were selected from the SALK mutant library (Alonso *et al.*, 2003; http://signal.salk.edu/cgi-bin/tdnaexpress) and obtained from the Nottingham Arabidopsis Stock Centre (NASC, http://arabidopsis.info).

[b] Maximum and minimum interaction phenotype (IP) scores observed among all plants tested for each mutant line. Plants were spray-inoculated with the *X. campestris* pv. *raphani* strain HRI8305. A total of 8 or 16 plants were tested for each homozygous or segregating line, respectively, in one experiment repeat. IPs were scored 10 days after inoculation using a six-point scale (see Chapter 2, Figure 4). The single mutant line that showed fully developed leaf spot symptoms (IP 3 to 5) is highlighted in yellow.

## APPENDIX 7. Genetic map of an *Arabidopsis thaliana* Col×Nd-1 inbred mapping population

The genetic map presented below was estimated based on the allelic scores of 98 $F_9$ Col×Nd-1 recombinant inbred lines for 103 markers spanning the genome of *A. thaliana* determined in previous investigations (Deslandes *et al.*, 1998; Werner *et al.*, 2005; E. Holub, pers. comm.) and the physical positions of each marker in the reference genome of *A. thaliana* accession Columbia. The genetic positions for the significant LOD peak and the 2-LOD interval associated with resistance to *Xanthomonas campestris* pv. *raphani* race 2 strain HRI8305 as predicted by statistical mapping analysis (Section 3.3.2), are indicated by a green dot and a green bar respectively. I to IV indicate the five chromosomes of *A. thaliana.* For each chromosome, the marker names are indicated on the right side and the genetic distances (cM, centimorgan) are indicated on left side.



# APPENDIX 8. Genotype and phenotype data for six *Arabidopsis thaliana* $F_8$ recombinant inbred lines derived from a cross Oy-0×Col-0 that have been spray-inoculated with *Xanthomonas campestris* pv. *raphani* race 2 strain HRI8305

| | | Genotype at genetic marker[a] (position in Mb) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Chromosome 3[b] | | | | Chromosome 5[c] | | | | | | |
| **Plant line** | **IP[d]** | c3_19628 (19,63) | c3_20729 (20.73) | ***RXCR1***[b] (21.39) | c3_23412 (23.41) | c5_14766 (14.77) | c5_17570 (17.57) | c5_19316 (19.32) | c5_2031[illegible] (20.32) | c5_21319 (21.32) | c5_2241[illegible] (22.41) | c5_24997 (25.00) |
| **Parents** | | | | | | | | | | | | |
| Oy | R [0] | OO | OO | OO | OO | OO | OO | OO | OO | OO | OO | OO |
| Col | R [0] | CC | CC | CC | CC | CC | CC | CC | CC | CC | CC | CC |
| **$F_8$ lines** | | | | | | | | | | | | |
| 7 | R [1] | OO | OO | OO | OO | OC | CC | CC | CC | CC | CC | CC |
| 78 | R [1] | OO | OO | OO | OO | CC | OO | OO | OO | OO | OO | CC |
| 219 | R [1-2] | OO | OO | OO | OO | CC | CC | CC | CC | CC | CC | CC |
| 167 | R [2] | OO | OO | OO | OO | CC | OO | CC | CC | CC | CC | CC |
| 107 | S [3] | OO | OO | OO | OO | OO | CC | CC | CC | OO | OO | OO |
| 127 | S [3-4] | OO | OO | OO | OO | CC | CC | CC | CC | CC | CC | OO |
| **Control** | | | | | | | | | | | | |
| Nd-1 | S [4] | na | na | na | na | na | na | na | na | na | na | na |

Abbreviations: CC, homozygous for the Col-0 allele (highlighted in brown), OO, homozygous for the Oy-0 allele (highlighted in blue) and OC, heterozygous (highlighted in grey); IP, interaction phenotype; na, not available; R, resistant; S, susceptible.

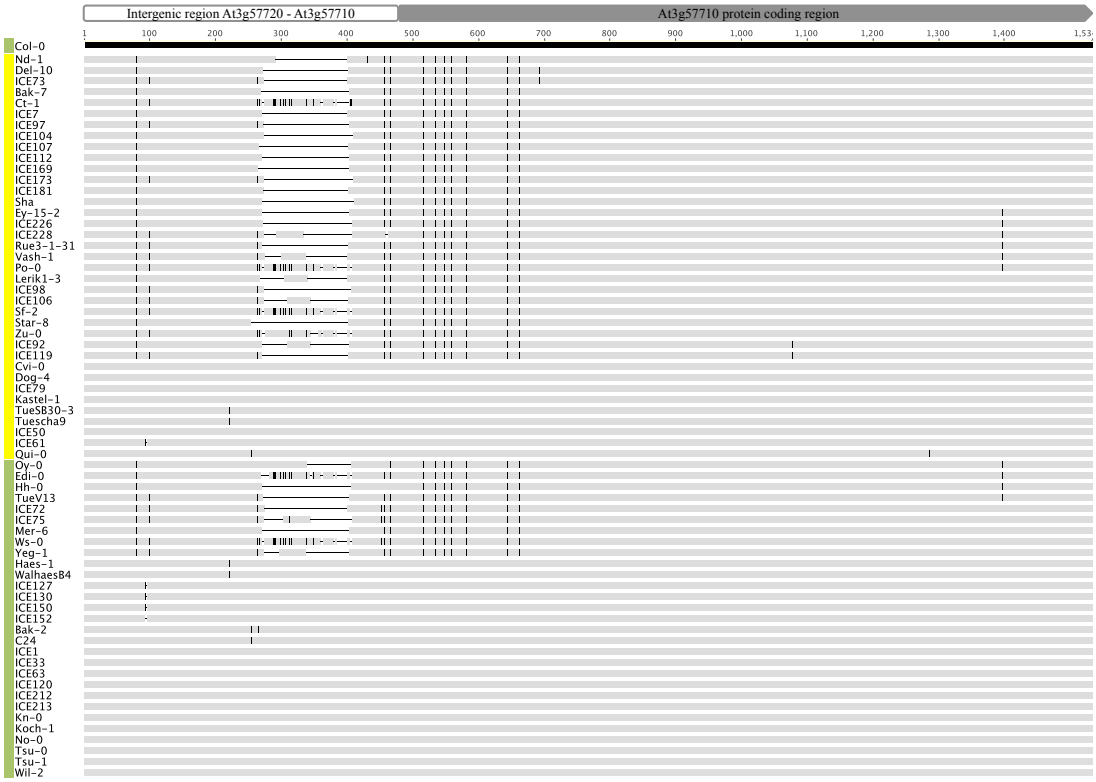
[a] Genotype data available at the Versailles Arabidopsis Stock Centre (http://publiclines.versailles.inra.fr; Simon *et al.*, 2008).

[b] Molecular markers spanning the map interval of *RXCR1* locus (20,256,936 - 23,291,586 bp) that was predicted by interval mapping analysis using MAGIC lines (Section 3.3.1, Chapter 3). The position in the chromosome for *RXCR1* locus is indicated according to the position of the gene (At3g57710) identified in this study conferring resistance to *X. campestris* pv. *raphani* race 2 (strain HRI8305). The genotypes for *RXCR1* locus were predicted based on the genotypes of the neighbouring markers.

[c] Molecular markers spanning the map interval of *RXCR2* locus (16,429,063 - 22,697,401 bp) that was predicted by interval mapping analysis using MAGIC lines (Section 3.3.1, Chapter 3).

[d] Minimum and maximum IP scores of *A. thaliana* plants spray-inoculated with *X. campestris* pv. *raphani* race 2 (strain HRI8305) (four plants tested per line in one experiment). The six-point scale defined in this study (Figure 4, Chapter 2) was used to assess the IPs. Plant lines were considered: resistant (R) if the IP observed in four plants tested was scored from 0 to 2, and susceptible (S) if the IP observed in four plants was scored from 3 to 5. The accession Nd-1 was used as a susceptible control.

**APPENDIX 9. Sequence polymorphisms in the *RXCR1* gene (At3g57710) and the intergenic region between *RXCR1* and AT3g57720 genes, of a collection of resistant and susceptible *Arabidopsis thaliana* accessions to *Xanthomonas campestris* pv. *raphani* (race 2, strain HRI8305)**




Green and yellow bars indicate, respectively, resistant and susceptible *A. thaliana* accessions to *X. campestris* pv. *raphani* race 2 (strain HRI8305) identified in this study (Tables 6 and 8, Chapter 2). All nucleotide sequences compared were retrieved from the *Arabidopsis* 1001 genomes database (http://signal.salk.edu/atg1001/3.0/gebrowser.php). Sequence variation is indicated in comparison to the reference sequence of accession Col-0. Vertical bars represent nucleotide changes, horizontal bars represent nucleotide deletions and grey bars indicate conserved sequences. The figure presented was created using Geneious software (Kearse *et al.*, 2012).

# APPENDIX 10. List of *16S* and *gyrB* gene sequences obtained in this study or retrieved from the NCBI nucleotide database

| Gene | Strain | | Sequence length (nt) | NCBI accession[a] | Reference |
|---|---|---|---|---|---|
| | Description | Accession | | | |
| *16S* | *Xanthomonas albilineans* | LMG494$^{T}$ | 1500 | X95918 | Hauben *et al.* (1997) |
| *16S* | *X. alfalfae* | LMG495$^{T}$ | 1499 | NR_104957 | Yarza *et al.* (2013) |
| *16S* | *X. arboricola* | LMG747$^{T}$ | 1502 | Y10757 | Hauben *et al.* (1997) |
| *16S* | *X. axonopodis* | LMG538$^{T}$ | 1502 | X95919 | Hauben *et al.* (1997) |
| *16S* | *X. bromi* | LMG947$^{T}$ | 1502 | Y10764 | Hauben *et al.* (1997) |
| *16S* | *X. campestris* | HRI8506 | 1362 | na | This study |
| *16S* | *X. campestris* | HRI8507 | 1343 | na | This study |
| *16S* | *X. campestris* | HRI8514 | 1337 | na | This study |
| *16S* | *X. campestris* | HRI8516A | 1379 | na | This study |
| *16S* | *X. campestris* | HRI8806 | 1370 | na | This study |
| *16S* | *X. campestris* | HRI8807 | 1367 | na | This study |
| *16S* | *X. campestris* | HRI8808 | 1368 | na | This study |
| *16S* | *X. campestris* | HRI8821 | 1356 | na | This study |
| *16S* | *X. campestris* | HRI8823 | 1378 | na | This study |
| *16S* | *X. campestris* | HRI8824A | 1368 | na | This study |
| *16S* | *X. campestris* | HRI8825 | 1378 | na | This study |
| *16S* | *X. campestris* | HRI8826A | 1369 | na | This study |
| *16S* | *X. campestris* | HRI8827A | 1382 | na | This study |
| *16S* | *X. campestris* pv. *campestris* race 3 | ATCC33913$^{T, Pt, Rt}$ | 1547 | NR_074936 | da Silva *et al.* (2002) |
| *16S* | *X. campestris* pv. *campestris* race 4 | HRI1279A$^{Rt}$ | 1334 | na | This study |
| *16S* | *X. campestris* pv. *campestris* race 5 | HRI3880$^{Rt}$ | 1331 | na | This study |
| *16S* | *X. campestris* pv. *campestris* race 6 | HRI6181$^{Rt}$ | 1349 | na | This study |
| *16S* | *X. campestris* pv. *raphani* race 2 | HRI8305$^{Rt}$ | 1344 | na | This study |
| *16S* | *X. campestris* pv. *raphani* race 3 | 756C | 1535 | CP002789 (locus 480035-481569) | Bogdanove *et al.* (2011) |
| *16S* | *X. cassavae* | CFBP4642$^{T}$ | 1502 | Y10762 | Hauben *et al.* (1997) |
| *16S* | *X. citri* | LMG9322$^{T}$ | 1499 | NR_104964 | Yarza *et al.* (2013) |
| *16S* | *X. codiaei* | LMG8678$^{T}$ | 1502 | Y10765 | Hauben *et al.* (1997) |
| *16S* | *X. cucurbitae* | LMG690$^{T}$ | 1502 | Y10760 | Hauben *et al.* (1997) |
| *16S* | *X. cynarae* | CFBP4188$^{T}$ | 1552 | AF208315 | Trebaol *et al.* (2000) |
| *16S* | *X. dyei* | ICMP12167$^{T}$ | 1465 | NR_104949 | Yarza *et al.* (2013) |
| *16S* | *X. euvesicatoria* | DSM19128$^{T}$ | 1535 | NR_104773 | Yarza *et al.* (2013) |
| *16S* | *X. fragariae* | LMG708$^{T}$ | 1502 | X95920 | Hauben *et al.* (1997) |
| *16S* | *X. fuscans* | LMG826$^{T}$ | 1499 | NR_104958 | Yarza *et al.* (2013) |
| *16S* | *X. gardneri* | DSM19127$^{T}$ | 1537 | NR_104793 | Yarza *et al.* (2013) |
| *16S* | *X. hortorum* | LMG733$^{T}$ | 1502 | Y10759 | Hauben *et al.* (1997) |
| *16S* | *X. hyacinthi* | LMG739$^{T}$ | 1500 | Y10754 | Hauben *et al.* (1997) |
| *16S* | *X. melonis* | LMG8670$^{T}$ | 1500 | Y10756 | Hauben *et al.* (1997) |
| *16S* | *X. oryzae* | LMG5047$^{T}$ | 1502 | X95921 | Moore *et al.* (1997) |
| *16S* | *X. perforans* | DSM18975$^{T}$ | 1534 | NR_104792 | Yarza *et al.* (2013) |
| *16S* | *X. pisi* | LMG847$^{T}$ | 1502 | Y10758 | Hauben *et al.* (1997) |
| *16S* | *X. populi* | LMG5743$^{T}$ | 1475 | X95922 | Moore *et al.* (1997) |
| *16S* | *X. sacchari* | LMG471$^{T}$ | 1500 | Y10766 | Hauben *et al.* (1997) |
| *16S* | *X. theicola* | LMG8684$^{T}$ | 1502 | Y10763 | Hauben *et al.* (1997) |
| *16S* | *X. vasicola* | LMG736$^{T}$ | 1502 | Y10755 | Hauben *et al.* (1997) |
| *16S* | *X. translucens* | LMG876$^{T}$ | 1500 | X99299 | Hauben *et al.* (1997) |

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APPENDIX 10 (continued)

| Gene | Strain | | Sequence length (nt) | NCBI accession[a] | Reference |
|---|---|---|---|---|---|
| | Description | Accession | | | |
| *16S* | *X. vesicatoria* | ATCC35937$^{T}$ | 1502 | Y10761 | Hauben *et al.* (1997) |
| *16S* | *Pseudomonas* sp. | HRI8822A | 1359 | na | This study |
| *16S* | *Pseudomonas* sp. | HRI8822B | 1383 | na | This study |
| *16S* | *Pseudomonas* sp. | HRI8824B | 1365 | na | This study |
| *16S* | *Pseudomonas* sp. | HRI8827C | 1360 | na | This study |
| *16S* | *P. aeruginosa* | LMG1242$^{T}$ | 1491 | Z76651 | Moore *et al.* (1996) |
| *16S* | *P. argentinensis* | CH01$^{T}$ | 1529 | AY691188 | Peix *et al.* (2005) |
| *16S* | *P. asturiensis* | LPPA221$^{T}$ | 1502 | FM865870 | Gonzalez *et al.* (2012) |
| *16S* | *P. cannabina* pv. *cannabina* | CFBP2341$^{T, Pt}$ | 1531 | AJ492827 | Behrendt *et al.* (2003) |
| *16S* | *P. cannabina* pv. *alisalensis* | CFBP6866$^{Pt}$ | 1328 | GQ470207 | Bull *et al.* (2010b) |
| *16S* | *P. caricapapayae* | ATCC33615$^{T}$ | 1456 | D84010 | Anzai *et al.* (1997) |
| *16S* | *P. chlororaphis* | DSM50083$^{T}$ | 1493 | Z76657 | Moore *et al*. (1996) |
| *16S* | *P. cichorii* | LMG2162$^{T}$ | 1494 | Z76658 | Moore *et al.* (1996) |
| *16S* | *P. congelans* | DSM14939$^{T}$ | 1531 | AJ492828 | Behrendt *et al.* (2003) |
| *16S* | *P. flavescens* | B62$^{T}$ | 1529 | U01916 | Hildebrand *et al.* (1994) |
| *16S* | *P. fluorescens* | DSM50090$^{T}$ | 1507 | Z76662 | Moore *et al.* (1996) |
| *16S* | *P. fulva* | NRIC0180$^{T}$ | 1484 | AB060136 | Uchino *et al.* (2001) |
| *16S* | *P. meliae* | CCUG51503$^{T}$ | 1512 | HF558390 | Yarza *et al.* (2013) |
| *16S* | *P. parafulva* | AJ2129$^{T}$ | 1484 | AB060132 | Uchino *et al.* (2001) |
| *16S* | *P. pertucinogena* | IFO14163$^{T}$ | 1505 | AB021380 | Anzai *et al.* (2000) |
| *16S* | *P. punonensis* | LMG26839$^{T}$ | 1532 | JQ344321 | Ramos *et al.* (2013) |
| *16S* | *P. putida* | DSM291$^{T}$ | 1492 | Z76667 | Moore *et al.* (1996) |
| *16S* | *P. savastanoi* | ATCC13522$^{T}$ | 1502 | AB021402 | Anzai *et al*. (1997) |
| *16S* | *P. straminea* | IAM1598$^{T}$ | 1527 | D84023 | Anzai *et al.* (1997) |
| *16S* | *P. stutzeri* | CCUG11256$^{T}$ | 1456 | U26262 | Bennasar *et al.* (1996) |
| *16S* | *P. syringae* pv. *syringae* | LMG1247$^{T, Pt}$ | 1494 | Z76669 | Moore *et al.* (1996) |
| *16S* | *P. syringae* pv. *maculicola* | CFBP1657$^{Pt}$ | 1328 | GQ470210 | Bull *et al.* (2010b) |
| *16S* | *P. tremae* | CFBP6111$^{T}$ | 1531 | AJ492826 | Behrendt *et al.* (2003) |
| *16S* | *P. viridiflava* | LMG2352$^{T}$ | 1492 | Z76671 | Moore *et al.* (1996) |
| *16S* | *Stenotrophomonas maltophilia* | LMG958$^{T}$ | 1500 | X95923 | Moore *et al.* (1997) |
| *gyrB* | *X. albilineans* | ICMP196$^{T}$ | 865 | EU498963 | Young *et al.* (2008) |
| *gyrB* | *X. alfalfae* | LMG495$^{T}$ | 530 | EU007542 | Parkinson *et al.* (2007) |
| *gyrB* | *X. arboricola* | NCPPB411$^{T}$ | 775 | FN667772 | Marcelletti *et al.* (2010) |
| *gyrB* | *X. axonopodis* | ICMP50$^{T}$ | 865 | EU498952 | Young *et al*. (2008) |
| *gyrB* | *X. bromi* | ICMP12545$^{T}$ | 865 | EU499052 | Young *et al*. (2008) |
| *gyrB* | *X. campestris* | HRI8506 | 803 | na | This study |
| *gyrB* | *X. campestris* | HRI8507 | 806 | na | This study |
| *gyrB* | *X. campestris* | HRI8514 | 794 | na | This study |
| *gyrB* | *X. campestris* | HRI8516A | 788 | na | This study |
| *gyrB* | *X. campestris* | HRI8806 | 797 | na | This study |
| *gyrB* | *X. campestris* | HRI8807 | 772 | na | This study |
| *gyrB* | *X. campestris* | HRI8808 | 816 | na | This study |
| *gyrB* | *X. campestris* | HRI8821 | 820 | na | This study |
| *gyrB* | *X. campestris* | HRI8823 | 542 | na | This study |
| *gyrB* | *X. campestris* | HRI8825 | 783 | na | This study |
| *gyrB* | *X. campestris* | NCPPB347$^{mpt}$ | 530 | EU285231 | Parkinson *et al.* (2009) |
| *gyrB* | *X. campestris* pv. *barbareae* | NCPPB983$^{Pt}$ | 530 | EU285239 | Parkinson *et al.* (2009) |
| *gyrB* | *X. campestris* pv. *campestris* race 1 | HRI3811$^{Rt}$ | 777 | HQ200308 | Mulema *et al.* (NCBI, 2010) |

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APPENDIX 10 (continued)

| Gene | Strain | | Sequence length (nt) | NCBI accession[a] | Reference |
|---|---|---|---|---|---|
| | Description | Accession | | | |
| *gyrB* | *X. campestris* pv. *campestris* race 2 | HRI3849$^{Rt}$ | 768 | HQ200320 | Mulema *et al.* (NCBI, 2010) |
| *gyrB* | *X. campestris* pv. *campestris* race 3 | ATCC33913$^{T, Pt, Rt}$ | 2445 | AE008922 (locus 4853-7297) | da Silva *et al.* (2002) |
| *gyrB* | *X. campestris* pv. *campestris* race 4 | HRI1279A$^{Rt}$ | 798 | na | This study |
| *gyrB* | *X. campestris* pv. *campestris* race 5 | HRI3880$^{Rt}$ | 782 | na | This study |
| *gyrB* | *X. campestris* pv. *campestris* race 6 | HRI6181$^{Rt}$ | 800 | na | This study |
| *gyrB* | *X. campestris* pv. *campestris* race 7 | HRI8450A | 785 | HQ200373 | Mulema *et al.* (NCBI, 2010) |
| *gyrB* | *X. campestris* pv. *campestris* race 9 | LMG8004$^{Rt}$ | 2445 | CP000050 (locus 4853-7297) | Qian *et al.* (2005) |
| *gyrB* | *X. campestris* pv. *incanae* | NCPPB937$^{Pt}$ | 530 | EU285236 | Parkinson *et al.* (2009) |
| *gyrB* | *X. campestris* pv. *plantaginis* | NCPPB1061$^{Pt}$ | 530 | EU285218 | Parkinson *et al.* (2009) |
| *gyrB* | *X. campestris* pv. *raphani* race 1 | NCPPB1946$^{Pt}$ | 530 | EU285222 | Parkinson *et al.* (2009) |
| *gyrB* | *X. campestris* pv. *raphani* race 1 | HRI6490$^{Rt}$ | 777 | HQ200345 | Mulema *et al.* (NCBI, 2010) |
| *gyrB* | *X. campestris* pv. *raphani* race 2 | HRI8305$^{Rt}$ | 786 | na | This study |
| *gyrB* | *X. campestris* pv. *raphani* race 3 | HRI6519$^{Rt}$ | 786 | HQ200347 | Mulema *et al.* (NCBI, 2010) |
| *gyrB* | *X. cassavae* | ICMP204$^{T}$ | 865 | EU498965 | Young *et al.* (2008) |
| *gyrB* | *X. citri* | LMG9322$^{T}$ | 530 | EU007540 | Parkinson *et al.* (2007) |
| *gyrB* | *X. codiaei* | ICMP9513$^{T}$ | 865 | EU499038 | Young *et al.* (2008) |
| *gyrB* | *X. cucurbitae* | ICMP2299$^{T}$ | 865 | EU498989 | Young *et al.* (2008) |
| *gyrB* | *X. cynarae* | ICMP16775$^{T}$ | 865 | EU499061 | Young *et al.* (2008) |
| *gyrB* | *X. dyei* | ICMP12167$^{T}$ | 865 | GQ183098 | Young *et al.* (2010) |
| *gyrB* | *X. euvesicatoria* | ICMP109$^{T}$ | 701 | GQ461738 | Myung *et al.* (NCBI, 2009) |
| *gyrB* | *X. fragariae* | ICMP5715$^{T}$ | 865 | EU499000 | Young *et al.* (2008) |
| *gyrB* | *X. fuscans* | NCPPB381$^{T}$ | 530 | EU007541 | Parkinson *et al.* (2007) |
| *gyrB* | *X. gardneri* | ICMP16689$^{T}$ | 865 | EU499058 | Young *et al.* (2008) |
| *gyrB* | *X. hortorum* | ICMP453$^{T}$ | 865 | EU498975 | Young *et al.* (2008) |
| *gyrB* | *X. hyacinthi* | ICMP189$^{T}$ | 865 | EU498960 | Young *et al.* (2008) |
| *gyrB* | *X. melonis* | ICMP8683 | 865 | EU499032 | Young *et al.* (2008) |
| *gyrB* | *X. oryzae* | ICMP3125$^{T}$ | 864 | EU498993 | Young *et al.* (2008) |
| *gyrB* | *X. perforans* | ICMP16690$^{T}$ | 865 | EU499059 | Young *et al.* (2008) |
| *gyrB* | *X. pisi* | ICMP570$^{T}$ | 865 | EU498976 | Young *et al.* (2008) |
| *gyrB* | *X. populi* | ICMP5816$^{T}$ | 864 | EU499014 | Young *et al.* (2008) |
| *gyrB* | *X. sacchari* | ICMP16916$^{T}$ | 864 | EU499063 | Young *et al.* (2008) |
| *gyrB* | *X. theicola* | ICMP6774$^{T}$ | 865 | EU499020 | Young *et al.* (2008) |
| *gyrB* | *X. translucens* | ICMP5752$^{T}$ | 865 | EU499009 | Young *et al.* (2008) |
| *gyrB* | *X. vasicola* | ICMP3103$^{T}$ | 865 | EU498992 | Young *et al.* (2008) |
| *gyrB* | *X. vesicatoria* | ICMP63$^{T}$ | 865 | EU498954 | Young *et al.* (2008) |
| *gyrB* | *Stenotrophomonas maltophilia* | R551-3 | 2460 | CP001111 (locus 5128-7587) | Lucas *et al.* (NCBI, 2008) |

Abbreviations: HRI, formerly Horticulture Research International, now University of Warwick Crop Centre, Wellesbourne, UK; na, Not available; NCBI, National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov); nt, nucleotide; mpt, misidentified pathotype of *X. campestris* pv. *armoraciae* (Vicente *et al.*, 2006; Fargier & Manceau, 2007); Pt, pathotype strain; Rt, race-type strain; T, species type strain.

[a] Accession of sequences retrieved from the nucleotide database of NCBI (http://www.ncbi.nlm.nih.gov).

# APPENDIX 11. Top hits of BLASTN searches of *16S* gene sequences of bacterial strains isolated from brassicas grown in Mauritius, against the nucleotide database of NCBI

| HRI strain accession | Sequence length (nt) | Description [a] | Id (%) | Qc (%) | E-value | Score |
|---|---|---|---|---|---|---|
| ***Xanthomonas* strains** | | | | | | |
| 8506 | 1362 | *Xanthomonas* spp. (*arboricola*, *campestris*, *cynarae*, *gardeneri* and *hortorum*) | 100.0 | 100.0 | 0.0 | 2457.5 |
| 8507 | 1343 | *Xanthomonas* spp. (*arboricola*, *campestris*, *cynarae*, *gardeneri* and *hortorum*) | 100.0 | 100.0 | 0.0 | 2423.2 |
| 8514 | 1337 | *Xanthomonas* spp. (*arboricola*, *campestris*, *cynarae*, *gardeneri* and *hortorum*) | 100.0 | 100.0 | 0.0 | 2412.4 |
| 8516A | 1379 | *Xanthomonas* spp. (*arboricola*, *campestris*, *cynarae*, *gardeneri* and *hortorum*) | 100.0 | 100.0 | 0.0 | 2488.1 |
| 8821 | 1356 | *Xanthomonas* spp. (*arboricola*, *campestris*, *cynarae*, *gardeneri* and *hortorum*) | 100.0 | 100.0 | 0.0 | 2446.7 |
| 8823 | 1378 | *Xanthomonas* spp. (*arboricola*, *campestris*, *cynarae*, *gardeneri* and *hortorum*) | 100.0 | 100.0 | 0.0 | 2486.3 |
| 8824A | 1368 | *Xanthomonas* spp. (*arboricola*, *campestris*, *cynarae*, *gardeneri* and *hortorum*) | 100.0 | 100.0 | 0.0 | 2468.3 |
| 8825 | 1378 | *Xanthomonas* spp. (*arboricola*, *campestris*, *cynarae*, *gardeneri* and *hortorum*) | 100.0 | 100.0 | 0.0 | 2486.3 |
| 8826A | 1368 | *Xanthomonas* spp. (*arboricola*, *campestris*, *cynarae*, *gardeneri* and *hortorum*) | 100.0 | 99.9 | 0.0 | 2468.3 |
| 8827A | 1382 | *Xanthomonas* spp. (*arboricola*, *campestris*, *cynarae*, *gardeneri* and *hortorum*) | 100.0 | 100.0 | 0.0 | 2493.5 |
| ***Pseudomonas* strains** | | | | | | |
| 8822A | 1358 | *Pseudomonas argentinensis* | 100.0 | 99.9 | 0.0 | 2450.3 |
| 8822B | 1383 | *P. cichorii* | 99.9 | 100.0 | 0.0 | 2491.7 |
| 8824B | 1365 | *P. cichorii* | 100.0 | 100.0 | 0.0 | 2462.9 |
| 8827B | 1360 | *P. viridiflava* | 100.0 | 100.0 | 0.0 | 2453.9 |
| 8827C | 1360 | *P. viridiflava* | 100.0 | 100.0 | 0.0 | 2453.9 |

Abbreviations: HRI, formerly Horticulture Research International, now University of Warwick Crop Centre, Wellesbourne, UK; Id, sequence identity; nt, nucleotide; Qc, query coverage.

[a] The results shown for each strain are the hits with the highest score obtained by similarity searches against the nucleotide database of NCBI (http://www.ncbi.nlm.nih.gov) using the BLASTN program (Altschul *et al.*, 1990).

**APPENDIX 12. Phylogenetic tree of *16S* gene sequences of *Xanthomonas* strains isolated from brassicas grown in Mauritius and South Carolina and strains of *Xanthomonas* species and *X. campestris* pathovars**

APPENDIX 12 (continued)

The phylogenetic tree was constructed using the Maximum Likelihood method (Felsenstein, 1981) based on the Hasegawa-Kishino-Yano model (Hasegawa *et al.*, 1985) and it was rooted on *S. maltophilia*. Bootstrap values ≥ 50% from 1000 replicates are shown above the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 47 nucleotide sequences and 1303 nucleotide positions, and it was conducted in MEGA6 software (Tamura *et al*., 2013). Strain accessions are indicated. T, Pt and Rt, indicate type strains of species, pathovars and races, respectively.

# APPENDIX 13. Genomic sequence reads obtained for the 32 newly sequenced *Xanthomonas campestris* strains under study

| Strain | | Sequencing type | Sequencing service | No. of reads | Average read length (bp) | Depth of coverage | Source |
|---|---|---|---|---|---|---|---|
| Race | HRI accession | | | | | | |
| ***Xanthomonas campestris* pv. *raphani*** | | | | | | | |
| 1 | 6490 | Paired-end | Warwick | 7,966,276 | 67.9 | 108 | This study |
| 1 | 8298 | Paired-end | Warwick | 9,792,660 | 67.9 | 133 | This study |
| 1 | 8803 | Paired-end | Exeter | 2,580,298 | 234 | 121 | This study |
| 2 | 6520 | Paired-end | Exeter | 973,760 | 222.3 | 43 | This study |
| 2 | 8305 | Paired-end | Warwick | 24,700,418 | 53.4 | 264 | E. Holub and J. Vicente |
| 2 | 8474 | Paired-end | Warwick | 8,472,342 | 67.9 | 115 | This study |
| 3 | 6519 | Paired-end | Warwick | 8,242,692 | 67.8 | 112 | This study |
| 3 | 8299 | Paired-end | Exeter | 863,188 | 231.5 | 40 | This study |
| ***X. campestris* pv. *campestris*** | | | | | | | |
| 1 | 3811 | Paired-end | Warwick | 16,888,122 | 35 | 118 | E. Holub and J. Vicente |
| 1 | 6195 | Paired-end | Exeter | 1,056,916 | 224.9 | 48 | This study |
| 1 | 8333 | Paired-end | Exeter | 1,206,438 | 228.3 | 55 | This study |
| 2 | 3849A | Single-end | Exeter | 2,248,430 | 53.1 | 24 | D. Studholme and M. Grant |
| 3 | 8819 | Paired-end | Exeter | 553,722 | 234.9 | 26 | This study |
| 4 | 1279A | Single-end | Exeter | 1,844,367 | 53.2 | 20 | D. Studholme and M. Grant |
| 4 | 6312A | Paired-end | Exeter | 1,195,630 | 228.8 | 55 | This study |
| 4 | 7758 | Paired-end | Exeter | 1,001,228 | 222.9 | 45 | This study |
| 4 | 7806 | Paired-end | Exeter | 979,058 | 225.1 | 44 | This study |
| 4 | 8506 | Paired-end | Warwick | 51,493,870 | 65.2 | 671 | This study |
| 4 | 8821 | Paired-end | Exeter | 1,071,088 | 230.9 | 49 | This study |
| 5 | 3880 | Single-end | Exeter | 2,659,130 | 50.4 | 27 | D. Studholme and M. Grant |
| 5 | 6382 | Paired-end | Exeter | 1,280,180 | 227.4 | 58 | This study |
| 6 | 6181 | Paired-end | Exeter | 1,476,294 | 226.1 | 67 | This study |
| 6 | 6185 | Paired-end | Exeter | 1,055,652 | 228.6 | 48 | This study |
| 6 | 8450B | Paired-end | Exeter | 847,746 | 231.1 | 39 | This study |
| 6 | 8497 | Paired-end | Exeter | 1,329,838 | 233.4 | 62 | This study |
| 6 | 8806 | Paired-end | Warwick | 7,859,300 | 67.8 | 107 | This study |
| 7 | 8450A | Paired-end | Exeter | 1,376,102 | 231.6 | 64 | This study |
| 8 | 8815 | Paired-end | Exeter | 1,089,698 | 234.1 | 51 | This study |
| ***X. campestris* pv. *incanae*** (vascular pathogen of garden stock) | | | | | | | |
| | 6378 | Paired-end | Exeter | 5,228,136 | 66.8 | 37 | D. Studholme and M. Grant |
| ***X. campestris*** (vascular pathogen of wallflower) | | | | | | | |
| | 5219A | Paired-end | Exeter | 5,015,012 | 66.9 | 67 | D. Studholme and M. Grant |
| ***X. campestris*** (vascular pathogen of candytuft) | | | | | | | |
| | 6375 | Single-end | Exeter | 1,954,121 | 54.1 | 21 | D. Studholme and M. Grant |
| ***X. campestris* pv. *armoraciae*** (reported to be non-pathogenic) | | | | | | | |
| | 6376 | Paired-end | Exeter | 832,832 | 234.5 | 39 | This study |

# APPENDIX 14. *De novo* genome assemblies

| Strain | | Number | Contig size (bp) | | | | | G+C |
|---|---|---|---|---|---|---|---|---|
| Race | HRI accession | of contigs | Shortest | Median | Longest | N50 length [a] | Sum | content (%) |
| ***Xanthomonas campestris* pv. *raphani*** | | | | | | | | |
| 1 | 6490 | 3652 | 201 | 764 | 24,383 | 2,163 | 4,725,946 | 65.2 |
| 1 | 8298 | 1860 | 201 | 1,382 | 31,292 | 5,163 | 4,863,434 | 65.2 |
| 1 | 8803 | 65 | 365 | 32,931 | 503,233 | 180,118 | 4,927,478 | 65.3 |
| 2 | 6520 | 265 | 325 | 11,568 | 214,183 | 37,519 | 5,004,832 | 65.3 |
| 2 | 8305 | 1693 | 200 | 1,643 | 25,294 | 5,276 | 4,775,942 | 65.2 |
| 2 | 8474 | 656 | 202 | 4,602 | 63,027 | 14,229 | 4,874,916 | 65.3 |
| 3 | 6519 | 750 | 200 | 3,374 | 71,422 | 13,469 | 4,866,628 | 65.4 |
| 3 | 8299 | 174 | 317 | 12,644 | 232,126 | 65,380 | 4,957,806 | 65.3 |
| ***X. campestris* pv. *campestris*** | | | | | | | | |
| 1 | 3811 | 1276 | 202 | 2,152 | 45,698 | 7,359 | 4,923,466 | 64.9 |
| 1 | 6195 | 162 | 325 | 11,637 | 296,747 | 80,605 | 4,997,176 | 65.1 |
| 1 | 8333 | 191 | 333 | 10,333 | 183,781 | 75,565 | 5,061,065 | 65.1 |
| 3 | 8819 | 699 | 285 | 1,997 | 136,591 | 20,590 | 5,172,259 | 65.0 |
| 4 | 6312A | 179 | 325 | 6,476 | 311,343 | 87,255 | 5,088,915 | 65.0 |
| 4 | 7758 | 318 | 317 | 6,975 | 235,716 | 36,320 | 5,088,379 | 65.1 |
| 4 | 7806 | 259 | 317 | 8,668 | 136,924 | 49,253 | 5,111,785 | 65.0 |
| 4 | 8506 | 2635 | 201 | 770 | 23,098 | 3,767 | 4,556,594 | 65.8 |
| 4 | 8821 | 296 | 325 | 6,156 | 155,288 | 50,013 | 5,344,066 | 64.8 |
| 5 | 6382 | 174 | 325 | 5,129 | 316,374 | 87,167 | 5,102,693 | 65.0 |
| 6 | 6181 | 156 | 333 | 5,321 | 330,504 | 117,902 | 5,041,193 | 65.1 |
| 6 | 6185 | 172 | 325 | 8,538 | 209,806 | 73,981 | 4,938,185 | 65.2 |
| 6 | 8450B | 301 | 309 | 8,224 | 140,548 | 39,029 | 5,023,762 | 65.2 |
| 6 | 8497 | 187 | 333 | 2,400 | 378,807 | 109,664 | 5,197,081 | 65.0 |
| 6 | 8806 | 5174 | 201 | 583 | 13,454 | 1,262 | 4,557,622 | 65.1 |
| 7 | 8450A | 158 | 333 | 8,003 | 271,334 | 102,359 | 4,989,618 | 65.2 |
| 8 | 8815 | 166 | 325 | 6,636 | 288,155 | 92,575 | 5,040,016 | 65.1 |
| ***X. campestris* pv. *incanae*** (vascular pathogen of garden stock) | | | | | | | | |
| | 6378 | 618 | 201 | 4,560 | 71,397 | 16,799 | 4,873,544 | 65.1 |
| ***X. campestris*** (vascular pathogen of wallflower) | | | | | | | | |
| | 5219A | 775 | 201 | 3,289 | 67,966 | 13,152 | 4,865,146 | 65.2 |
| ***X. campestris* pv. *armoraciae*** (reported to be non-pathogenic) | | | | | | | | |
| | 6376 | 268 | 325 | 9,749 | 188,051 | 42,244 | 5,134,100 | 65.0 |

[a] N50 length is defined as the length for which the collection of all contigs of that length or longer contains at least half of the sum of the lengths of all contigs.

## APPENDIX 15. Breadth of coverage of pathovar-associated pan-gene sequences from alignments of genomic sequence reads of all *Xanthomonas campestris* strains under study

**a)** Five pan-genes detected in *Xanthomonas campestris* pv. *raphani* strains (breadth of coverage >80%) and not detected in *X. campestris* pv. *campestris* (*Xcc*) strains (breadth of coverage <20% and BLASTN hits of pan-gene sequences against the genome assemblies of all *Xcc* strains under study showed maximum query coverage 9.91% and maximum sequence identity 100%)

| Strain | | Pan-gene no. (predicted gene product) | | | | |
|---|---|---|---|---|---|---|
| Race | Accession | 1 (XopAD) | 2 (YapH) | 3 (Alpha-beta hydrolase) | 4 (Transcriptional regulator) | 5 (Hypothetical protein) |
| ***Xanthomonas campestris* pv. *raphani*** | | | | | | |
| 1 | 6490 | 99 | 99 | 99 | 98 | 98 |
| 1 | 8298 | 99 | 99 | 99 | 98 | 95 |
| 1 | 8803 | 99 | 99 | 99 | 98 | 94 |
| 2 | 6520 | 99 | 99 | 99 | 96 | 98 |
| 2 | 8305 | 99 | 99 | 99 | 98 | 98 |
| 2 | 8474 | 99 | 99 | 97 | 98 | 98 |
| 3 | 6519 | 99 | 99 | 98 | 98 | 98 |
| 3 | 8299 | 99 | 99 | 99 | 98 | 96 |
| 3 | 756C | 99 | 99 | 99 | 97 | 91 |
| ***X. campestris* pv. *campestris*** | | | | | | |
| 1 | 3811 | 0 | 2 | 0 | 0 | 0 |
| 1 | 6195 | 7 | 7 | 0 | 0 | 0 |
| 1 | 8333 | 0 | 0 | 0 | 0 | 0 |
| 1 | B100 | 0 | 0 | 0 | 0 | 0 |
| 2 | 3849A | 3 | 0 | 0 | 0 | 0 |
| 3 | 8819 | 0 | 4 | 3 | 0 | 0 |
| 3 | ATCC33913 | 0 | 0 | 0 | 0 | 0 |
| 4 | 1279A | 2 | 0 | 0 | 0 | 0 |
| 4 | 6312A | 2 | 4 | 0 | 7 | 0 |
| 4 | 7758 | 3 | 0 | 0 | 0 | 0 |
| 4 | 7806 | 0 | 0 | 0 | 0 | 0 |
| 4 | 8506 | 0 | 0 | 0 | 0 | 0 |
| 4 | 8821 | 0 | 0 | 0 | 0 | 0 |
| 5 | 3880 | 0 | 0 | 0 | 0 | 0 |
| 5 | 6382 | 1 | 0 | 0 | 0 | 0 |
| 6 | 6181 | 2 | 0 | 0 | 0 | 0 |
| 6 | 6185 | 18 | 8 | 10 | 0 | 0 |
| 6 | 8450B | 0 | 0 | 0 | 0 | 0 |
| 6 | 8497 | 4 | 6 | 0 | 0 | 0 |
| 6 | 8806 | 3 | 6 | 11 | 10 | 19 |
| 7 | 8450A | 0 | 0 | 0 | 0 | 0 |
| 8 | 8815 | 0 | 0 | 0 | 0 | 0 |
| 9 | LMG8004 | 0 | 0 | 0 | 0 | 0 |
| ***X. campestris* pv. *incanae*** (vascular pathogen of garden stock) | | | | | | |
| | 6378 | 98 | 0 | 0 | 0 | 16 |
| ***X. campestris*** (vascular pathogen of wallflower) | | | | | | |
| | 5219A | 20 | 0 | 99 | 0 | 0 |
| ***X. campestris*** (vascular pathogen of candytuft) | | | | | | |
| | 6375 | 99 | 0 | 0 | 0 | 91 |
| ***X. campestris* pv. *armoraciae*** (maybe non-pathogenic) | | | | | | |
| | 6376 | 17 | 8 | 99 | 0 | 0 |

APPENDIX 15 (continued)

**b)** Seven pan-genes detected in *X. campestris* pv. *campestris* strains (breadth of coverage >80%) and not detected in *X. campestris* pv. *raphani* (*Xcr*) strains (breadth of coverage <20% and BLASTN hits of pan-gene sequences against the genome assemblies of all *Xcr* strains under study showed maximum query coverage 20.48% and maximum sequence identity 86.10%)

| Strain | | Pan-gene no. (predicted gene product) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Race | Accession | 1 (AvrBs2) | 2 (XopAM) | 3 (XopK) | 4 (XopQ) | 5 (Type I restriction enzyme M) | 6 (ATP-dependent DNA helicase) | 7 (Hypothetical protein) |
| ***Xanthomonas campestris* pv. *raphani*** | | | | | | | | |
| 1 | 6490 | 0.0 | 0.0 | 0.0 | 0.0 | 4.2 | 4.8 | 0.0 |
| 1 | 8298 | 2.9 | 1.0 | 2.4 | 0.0 | 0.0 | 0.0 | 0.0 |
| 1 | 8803 | 10.5 | 13.8 | 18.7 | 0.0 | 2.9 | 0.0 | 17.6 |
| 2 | 6520 | 12.8 | 11.9 | 0.0 | 0.0 | 13.0 | 3.3 | 0.0 |
| 2 | 8305 | 0.0 | 1.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 2 | 8474 | 0.0 | 1.0 | 4.6 | 0.0 | 0.0 | 0.0 | 0.0 |
| 3 | 6519 | 0.0 | 1.9 | 0.0 | 0.0 | 0.0 | 4.8 | 0.0 |
| 3 | 8299 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 18.3 | 0.0 |
| 3 | 756C | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| ***X. campestris* pv. *campestris*** | | | | | | | | |
| 1 | 3811 | 98.6 | 98.6 | 98.7 | 98.6 | 98.7 | 98.6 | 98.6 |
| 1 | 6195 | 98.6 | 98.7 | 98.7 | 98.6 | 98.6 | 98.7 | 98.6 |
| 1 | 8333 | 98.6 | 98.7 | 98.7 | 98.6 | 98.7 | 98.7 | 98.6 |
| 1 | B100 | 98.7 | 98.7 | 98.7 | 98.6 | 98.6 | 98.7 | 98.6 |
| 2 | 3849A | 98.6 | 98.7 | 98.7 | 98.6 | 95.0 | 98.5 | 98.6 |
| 3 | 8819 | 98.6 | 98.6 | 98.6 | 98.6 | 98.4 | 98.7 | 98.6 |
| 3 | ATCC33913 | 98.7 | 98.7 | 98.7 | 98.6 | 98.6 | 98.7 | 98.6 |
| 4 | 1279A | 98.2 | 98.7 | 98.7 | 98.6 | 80.5 | 98.4 | 98.6 |
| 4 | 6312A | 98.7 | 98.7 | 98.7 | 98.6 | 96.8 | 98.4 | 98.6 |
| 4 | 7758 | 98.6 | 98.7 | 98.7 | 98.6 | 92.3 | 83.6 | 98.6 |
| 4 | 7806 | 98.7 | 98.7 | 98.7 | 98.6 | 83.6 | 98.7 | 98.6 |
| 4 | 8506 | 98.7 | 98.7 | 98.7 | 98.6 | 86.1 | 98.6 | 98.6 |
| 4 | 8821 | 98.7 | 98.7 | 98.7 | 98.6 | 84.8 | 83.6 | 98.6 |
| 5 | 3880 | 98.6 | 98.7 | 98.7 | 98.6 | 98.6 | 98.7 | 98.6 |
| 5 | 6382 | 98.7 | 98.7 | 98.7 | 98.6 | 98.7 | 98.7 | 98.6 |
| 6 | 6181 | 98.7 | 98.7 | 98.7 | 98.6 | 98.7 | 98.7 | 98.6 |
| 6 | 6185 | 98.7 | 98.7 | 98.6 | 98.6 | 98.7 | 98.7 | 98.6 |
| 6 | 8450B | 98.7 | 98.7 | 98.7 | 98.6 | 98.7 | 98.7 | 98.6 |
| 6 | 8497 | 98.7 | 98.7 | 98.7 | 98.6 | 98.7 | 98.7 | 98.6 |
| 6 | 8806 | 98.7 | 98.7 | 98.7 | 98.6 | 98.7 | 98.7 | 97.8 |
| 7 | 8450A | 98.7 | 98.7 | 98.7 | 98.6 | 98.7 | 98.7 | 98.6 |
| 8 | 8815 | 98.7 | 98.7 | 98.7 | 98.6 | 98.6 | 98.7 | 98.6 |
| 9 | LMG8004 | 98.7 | 98.7 | 98.7 | 98.6 | 98.6 | 98.7 | 97.8 |
| ***X. campestris* pv. *incanae*** (vascular pathogen of garden stock) | | | | | | | | |
| | 6378 | 97.9 | 98.7 | 97.1 | 98.6 | 92.1 | 8.2 | 98.6 |
| ***X. campestris*** (vascular pathogen of wallflower) | | | | | | | | |
| | 5219A | 97.8 | 98.7 | 98.7 | 16.0 | 95.1 | 0.0 | 0.0 |
| ***X. campestris*** (vascular pathogen of candytuft) | | | | | | | | |
| | 6375 | 0.0 | 95.6 | 98.7 | 98.6 | 98.6 | 98.6 | 97.6 |
| ***X. campestris* pv. *armoraciae*** (reported to be non-pathogenic) | | | | | | | | |
| | 6376 | 98.4 | 98.7 | 31.6 | 0.0 | 20.9 | 23.5 | 0.0 |

# APPENDIX 16. Gene sequences

## Gene 3

Predicted gene product: alpha-beta hydrolase

Organism: *Xanthomonas campestris* pv. *raphani* strain HRI6519

Nucleotide sequence:

```
ATGCAACTGCGCCTCCTTGTTCTGCTGCTCTTGCTGATCTCACCCACTGT
  CTTCGGAGCAGATATGTCCCACGACGCGAATAATTTCTACACCAGCGACA
  AGGTGGCCCTGCAGCAGGTCACCTTCAAGAACCAGTACCAGATGACCGTG
  GCCGGCACGCTGTTCGTGCCCAAAGGCCTGGACACCGGCAAGCGCCATCC
  GGCCATTGTGGTCGGGCACCCGATGGGTGCGACCAAGGAGCAGAGCGCCA
  ACCTCTATGCCACCAAACTTGCCGAGCAGGGCTTTGTGACATTGTCGCTG
  GACCTGTCGTTCTGGGGCGCAAGCGAGGGCCAGCCACGCAATGCGGTGTC
  GCCGGATATTTATGCGGAAGACTTCAGCGCAGCGGTGGACTTCCTGGGCA
  CGCAGGCATTCGTGGACAGCGCGCAGATCGGCGCGCTGGGCATCTGCGGC
  AGTGGCAGCTTCGTGATCAGCGCGGCCAAGATCGACCCACGCATGCGCGC
  CATTGCCACGGTGAGCATGTACGACATGGGCGCGGCCAACCGCAACGGGC
  TGCGCCATGGGCTCAGCCTGGAACAACGCAAACAGATCATTGCCGCAGCT
  GCCAAGCAGCGCTCGGCCGAATTCAGCGGCGGGCCGGTGGAGTACACCGG
  CGGCACGGTGCTGGCGCTGACGAAGGACTCCAACCCGATCGAACGCGAGT
  TCTATGATTTCTACCGAACGCCGCGTGGCCAGTACACCCCGCAAGGCCAA
  TCGCCCGATCACACCACGCGCCCGACGCTGACCAGCAACGTCAAGTTCAT
  GAACTTCTACCCGTTCAACGATATTGCCACCATCTCGCCACGGCCGATGC
  TGTTCATTGCCGGCGAAAACGCGCATTCCATCGAGTTCAGCGAAGAAGCG
  TACCGTCTGGCCGGCGAACCCAAGCAACTGGTGATCGTGCCCAACGCCGG
  CCACGTGGACCTGTATGACCGCGTCGCGCTCATCCCGTTCGACACGCTGA
  CTGCGTTCTTCCGCAAGAATCTGCAGTAA
```

## Gene 4

Predicted gene product: transcriptional regulator

Organism: *Xanthomonas campestris* pv. *raphani* strain HRI6519

Nucleotide sequence:

```
GTGCCTGCGCAGGCCGACGCCGCGTTGGAGAGCAAGCAGGCGCGCATCAT
    GGACGCGGCGCTGCAGCTTTTCCTGCGCCATGGCTACCGCAAGGTGAGCA
    TGAGCGACATCGCCGAAGCGGCGCAGATGTCACGGCCGTCGCTGTATGCC
    GCGTTCGCCAACAAGGAATCGGTCTTTGCCGCGCAGGTGCAACGCCAGCG
    CATGCTGTGCGTGGCCGACACCGAACGCCGCGTGCGCGGCGACCAGGACC
    TGCGCACGCAGCTGCAGCACCTGTTCGATATCTGGGTACTGGAGCCGGTG
    GCCGCGGTGATCGACTCGGCGAACGGCATCGATCTGATCAGCAATTGCGG
    CGTGTATGCGCCCGGTGCACTGGACGATCTCTACAGCCACATGGAAGCAA
    AACTGGTGCAGGTGCTGCGCCCGCAGATGGGTGCCGACAGCGCGATGCCC
    GCGGCCGACCTGGCGTACATCCTGCGCCTGGCCACCACCAGCCTGAAGGC
    CTCGACCGATAACGCAGCGGTGCTGCGGCGTCTCATTGCCGGACTCATCA
    CCATGGCGCTGGCCACGGCCGGTGTGCGTGCACCGCAAGCCCCAGCAACG
    CCACGGCGCCCGGTGCGCAAGAAGCCCGCCGCGCGCAGTTGA
```

## Gene 5

Predicted gene product: hypothetical protein

Organism: *Xanthomonas campestris* pv. *raphani* strain HRI8474

Nucleotide sequence:

```
ATGACACAGGCGCGGGTGGCGCCGGCGGATGCGGATGCGGTGATGCTGTG
   GGTGCGATGGGTGCGGCCGGTACCGGGACCGCAGCGGGTTGTGCGCGCGC
   CGCAGGTGCTGGCGATGCAGCGCGCAATGCTGGTTCCGGAGCAGCCGGTG
   CTGCAGGTTGCGCTGTGGCGCTGGGCGACACTGCCGATGCAGGCGGCAAC
   GGCGCAGCGGCGGCGGCGGGTGCCGCGCTTGCCAATGACGCAGGTGCAAG
   CGGTGTCGCCATCGCTGCAGGTGCGGCGAAGGCACCGAGTGCCAGTAGCA
   CCGCATCACGCAGGTACCGCTGTAGAAGGTTGA
```

## Gene 13

Predicted gene product: ABC transporter, ATP-binding protein

Organism: *Xanthomonas campestris* pv. *raphani* strain HRI8803

Nucleotide sequence:

```
ATGCGCGCACTCGATGCTAAACACTATCTGTTGGAAATCAAGCTCAAGAG
   GAAAGGTCAGACCTTTGATCGCTATCCTTTTTCTCTTCCTGCAGTCCGTC
   ACTTGGGTACTTTGGAGCTGCATCCGAAGGTAACCTTTATCGTTGGTGAG
   AACGGGAGTGGTAAGTCCACGCTGCTGGAAGCCACTGCGGTTGCCTTTGG
   ATTCAACCCGGAAGGAGGCACCAAGAACTTCAATTTCCAGACCCGTGCCT
   CGCACTCGGAGTTGCATGAGTACTTGACGCTTGTGAAAGGGGTTGCGCGC
   GCCAAGGATGGATTCTTCTTACGGGCAGAGAGTTTCTTCAATCTTGCTAC
   CGAGATCGAGCGGTTGGATGAGGGGCCTTTCGGGGGGGCAATCATTGATT
   CCTATGGTGGAGTCTCGCTTCATCAGCAATCTCATGGCGAATCTTTCTTT
   GCGTTGATGATGAACCGTTTCGGTGGAAACGGTCTTTACATACTCGATGA
   GCCTGAAGCTGCGCTTTCGCCTCAACGGCAGCTGGCAATGATGTCGCGAC
   TGCACCAGTTGGTACAGTCGCGATCACAGTTCTTGATCGCTACTCACTCG
   CCAATTCTCATGGCGTACCCTGATGCTTGTATTTACCAGATCGGCGATAA
   TGGACTAGAGAAGGTTGGCTATGAGGATACGGAGCACTACATGGTTACCA
   AATATTTTCTCAACAACTACAAGCGCCAAATAGATCATTTGCTAGATTAG
```

## Gene 14

Predicted gene product: hypothetical protein

Organism: *Xanthomonas campestris* pv. *raphani* strain HRI8803

Nucleotide sequence:

```
ATGCGACAGCAGGTGGATGACTTGGAAGAGGATGTGGTGTCGGCGGCCAC
   GGATGGGAACGCGCACAACTGCGGTGAACTGGCGACCCTGGCCGTCCACT
   ATTTGCAGCAAGATCACAACCAGATCGCACGCCTTGCCTTCTTCAATGGA
   ACAGCGCACACCGCCGCGATCGTCGGCCCAGTGCCAGGCGCAGGCACCTT
   GCCTTCAGACATGACAGATTGGGACGCGGACATCTATGTGTGCGACCCCT
   GGTGCAATATCGCCTGCAGGGCAAACGACTATCCCGCCGAGTTCAAAGAA
   AAGATGGAAAAGTGGGACCGCGCCGGGAAACAGGTGTGGCTGTCAGGAAC
   TGGCTTCGTTTCCCCAACAAGCGATGAATGGATAAGCACAGTGCTTGGAG
   GAGAAAAGAAGGCCACATGA
```

## Gene 16

Predicted gene product: 3-oxoacyl-[acyl-carrier protein] reductase (oxidoreductase family)

Organism: *Xanthomonas campestris* pv. *campestris* strain HRI3811

Nucleotide sequence:

```
ATGAAGCACTTCTGCGACCGTGGCCACGGTGCCGTCGCAGCGCAATGTCG
   TGCGCCATCGTCACCACCTTCCGAACGCATGCCATGCAGGCGCACGCGTA
   TCGCCGATGTCTTTCTCTTGATTGAAGGAACCGCAATGCAACAGCAGACC
   GTTGTGCTGATCACGGGCGTGTCGTCCGGCATTGGCCGTGCCGCCGCAGA
   ACACTTCGCCCGAGCCGGCTGCCTCGTTTACGGCAGCGTGCGTCACCTGG
   CCGGTGCCACACCGTTGACGGCCGTGGAGCTGGTGGAGATGGATATCCGC
   GATGCCGCTTCGGTGCAGCGTGCGGTCGATGGCATCATCGCCCGCGCGGG
   CCGCATCGATGTGCTGGTCAACAACGCAGGCACCAATCTTGTCGGTGCCA
   TTGAAGAAACCAGCGTCGACGAAGCGGCCGCATTATTCGACATCAACCTA
   CTCGGCATCCTACGCACCGTGCAGGCCGTGCTGCCGCACATGCGTGCACG
   CGGCCAGGGCCGCATCGTCAATGTCAGCTCGGTGCTGGGGTTCCTGCCGG
   CGCCGTACATGGGCGTGTATGCCGCCTCCAAACATGCGGTGGAAGGGCTG
   TCGGAAACGCTGGATCACGAGCTGCGTCAATTCGGCATTCGCGTGACGCT
   GGTGGAGCCTGCGTATACGAAAACCAGCCTGGGCAGCAATTCGCCGGTGG
   TGCAGGCCACCATTGCCGATTACGACCGCGAGCGTGGCGTGGTGGCGCGG
   GCAGTCACGCACAGTATCGACACCGCGCCGGAACCGCATGGCGTGGCCGC
   CACCGTGGTGGAGGCGGCCCTGGGCAGGTGGCGCATGCGCCGCACACCCG
   CCGGGCAGGCATCGCTGCTGAGCAAGCTGCGCCGGTTCCTGCCCGCCAGC
   GCGGTGGACGGCAGCCTGCGCAAGCAGCTCGGGCTGCGGTGA
```

# APPENDIX 17. BLASTX top five hits plus the best hit to any type III effector protein, of pathovar-associated gene sequences against the RefSeq protein database of NCBI

| Gene no. [a] | BLASTX hits | | | | |
|---|---|---|---|---|---|
| | Protein name (RefSeq accession) | Organism name | Query coverage (%) | Identity (%) | E-value |
| ***Xcr*-associated genes** | | | | | |
| 1 | Type III effector protein XopAD (WP_014507096.1) | *Xanthomonas campestris* | 99 | 100 | 0 |
| | Type III effector protein XopAD (WP_044324910.1) | *Pseudomonas amygdali* | 98 | 66 | 0 |
| | Hypothetical protein (WP_015060666.1) | *P. savastanoi* | 98 | 65 | 0 |
| | Hypothetical protein (WP_015060647.1) | *P. savastanoi* | 98 | 70 | 0 |
| | Type III effector protein XopAD (WP_060410298.1) | *P. amygdali* | 98 | 69 | 0 |
| 2 | Transporter (WP_043921611.1) | *X. campestris* | 99 | 99 | 0 |
| | Transporter (WP_042596882.1) | *X. campestris* | 99 | 97 | 0 |
| | Transporter (WP_006448963.1) | *X. gardneri* | 99 | 87 | 0 |
| | Transporter (WP_039424255.1) | *X. vesicatoria* | 99 | 85 | 0 |
| | Transporter (WP_005991557.1) | *X. vesicatoria* | 99 | 85 | 0 |
| 3 | Membrane protein (WP_042597234.1) | *X. campestris* | 95 | 98 | 0 |
| | Hypothetical protein (WP_058562496.1) | *X. vesicatoria* | 95 | 91 | 0 |
| | Membrane protein (WP_039426692.1) | *X. vesicatoria* | 99 | 89 | 0 |
| | Hypothetical protein (WP_055853085.1) | *Xanthomonas* sp. | 96 | 89 | 0 |
| | Membrane protein (WP_046963499.1) | *X. pisi* | 95 | 88 | 0 |
| 4 | Transcriptional regulator (WP_043921655.1) | *X. campestris* | 89 | 97 | 2.00E-129 |
| | Transcriptional regulator (WP_042595084.1) | *X. campestris* | 89 | 96 | 3.00E-129 |
| | Transcriptional regulator (WP_058563319.1) | *X. vesicatoria* | 84 | 79 | 8.00E-96 |
| | Transcriptional regulator (WP_055819026.1) | *X. sp.* | 84 | 78 | 2.00E-95 |
| | Transcriptional regulator (WP_052318623.1) | *X. vesicatoria* | 84 | 79 | 3.00E-95 |
| 5 | Hypothetical protein (WP_014505980.1) | *X. campestris* | 91 | 94 | 2.00E-17 |
| | Hypothetical protein (WP_040606399.1) | *Salisaeta longa* | 43 | 33 | 7.4 |
| ***Xcc*-associated genes** | | | | | |
| 6 | Avirulence protein (WP_011035310.1) | *X. campestris* | 96 | 100 | 0 |
| | Avirulence protein (WP_012436888.1) | *X. campestris* | 96 | 99 | 0 |
| | Avirulence protein (WP_040940279.1) | *X. campestris* | 96 | 99 | 0 |
| | Avirulence protein (WP_047126487.1) | *X. arboricola* | 96 | 98 | 0 |
| | Avirulence protein (WP_047129835.1) | *X. arboricola* | 96 | 98 | 0 |
| | Type III effector AvrBs2 (WP_023901984.1) | *X. hortorum* | 96 | 89 | 0 |
| 7 | Transducer protein car (WP_011036289.1) | *X. campestris* | 99 | 100 | 0 |
| | Transducer protein car (WP_057673060.1) | *X. campestris* | 99 | 99 | 0 |
| | Type III secretion system effector (WP_012438997.1) | *X. campestris* | 99 | 99 | 0 |
| | Transducer protein car (WP_049798594.1) | *X. campestris* | 99 | 99 | 0 |
| | Hypothetical protein (WP_006452218.1) | *X. gardneri* | 99 | 99 | 0 |
| | Type III effector XopAM (WP_022559897.1) | *Xanthomonas* spp. | 96 | 70 | 0 |

*Continues next page*

APPENDIX 17 (continued)

| Gene no. a | BLASTX hits | | | | |
|---|---|---|---|---|---|
| | **Protein name (RefSeq accession)** | **Organism name** | **Query coverage (%)** | **Identity (%)** | **E-value** |
| 8 | Type III secretion system effector (WP_016944157.1) | *X. campestris* | 92 | 100 | 0 |
| | Type III secretion system effector (WP_040940889.1) | *X. campestris* | 92 | 99 | 0 |
| | Type III secretion system effector (WP_019237406.1) | *X. campestris* | 92 | 99 | 0 |
| | Hypothetical protein (WP_011038023.1) | *X. campestris* | 90 | 99 | 0 |
| | Type III secretion system effector (WP_039513347.1) | *X. arboricola* | 92 | 97 | 0 |
| | Putative XopK (WP_005988615.1) | *X. vesicatoria* | 89 | 61 | 0 |
| 9 | Hypothetical protein (WP_011036272.1) | *X. campestris* | 99 | 100 | 0 |
| | Type III effector (WP_050912382.1) | *X. campestris* | 99 | 99 | 0 |
| | Hypothetical protein (WP_011269952.1) | *X. campestris* | 99 | 99 | 0 |
| | Type III effector (WP_040941676.1) | *X. campestris* | 99 | 99 | 0 |
| | Type III effector (WP_043907627.1) | *X. gardneri* | 79 | 97 | 0 |
| | Type III effector XopQ (WP_022560457.1) | *Xanthomonas* spp. | 82 | 65 | 1.00E-164 |
| 10 | Type I restriction-modification protein subunit M (WP_011038026.1) | *X. campestris* | 99 | 100 | 0 |
| | Type I restriction-modification system, M subunit (WP_002813925.1) | *X. fragariae* | 99 | 97 | 0 |
| | Type I restriction endonuclease subunit M (WP_050912103.1) | *X. campestris* | 99 | 97 | 0 |
| | Type I restriction-modification protein subunit M (WP_006452953.1) | *X. gardneri* | 99 | 94 | 0 |
| | Type I restriction-modification protein subunit M (WP_039408830.1) | *X. campestris* | 99 | 96 | 0 |
| 11 | ATPase AAA (WP_011035773.1) | *X. campestris* | 99 | 100 | 0 |
| | AAA family ATPase (WP_050911218.1) | *X. campestris* | 99 | 99 | 0 |
| | DNA-binding protein (WP_003470501.1) | *X. translucens* | 99 | 90 | 0 |
| | Transcriptional regulator (WP_028322881.1) | *Desulfatiglans anilini* | 92 | 48 | 1.00E-127 |
| | Hypothetical protein (WP_058090657.1) | *Marinobacter* sp. | 94 | 50 | 4.00E-126 |
| 12 | Hypothetical protein (WP_011036273.1) | *X. campestris* | 99 | 100 | 6.00E-127 |
| | Hypothetical protein (WP_011269951.1) | *X. campestris* | 92 | 99 | 4.00E-126 |
| | Hypothetical protein (WP_006449287.1) | *X. gardneri* | 99 | 98 | 4.00E-124 |
| | Hypothetical protein (WP_023903000.1) | *X. hortorum* | 99 | 97 | 7.00E-121 |
| | Hypothetical protein (WP_043087353.1) | *X. arboricola* | 92 | 93 | 6.00E-116 |

Abbreviations: *Xcr*, *Xanthomonas campestris* pv. *raphani*; *Xcc*, *X. campestris* pv. *campestris*.

[a] The gene sequence used in BLASTX searches is described in Table 30 (Section 5.3.2, Chapter 5).

## APPENDIX 18. BLASTX top five hits of pathovar-associated gene sequences against predicted/known type III effector protein sequences of *Xanthomonas* spp. (listed in the *Xanthomonas* Resource database, http://www.xanthomonas.org/t3e.html)

| Gene no. [a] | BLASTX top hit | | | | |
|---|---|---|---|---|---|
| | Predicted/known type III effector (GenBank accession) | Organism name | Query coverage (%) | Identity (%) | E-value |
| ***Xcr*-associated genes** | | | | | |
| 1 | XopAD (AEL06362.1) | *Xanthomonas campestris* pv. *raphani* | 100 | 100 | 0 |
| | XopAD (ACD57041.1) | *X. oryzae* pv. *oryzae* | 86 | 61 | 0 |
| | XopAD (ZP_02245275.1) | *X. oryzae* pv. *oryzicola* | 86 | 63 | 0 |
| | XopAD (BAE70900.1) | *X. oryzae* pv. *oryzae* | 86 | 61 | 0 |
| | XopAD (AAW77655.1) | *X. oryzae* pv. *oryzae* | 86 | 61 | 0 |
| 2 | No hit | - | - | - | - |
| 3 | No hit | - | - | - | - |
| 4 | No hit | - | - | - | - |
| 5 | No hit | - | - | - | - |
| ***Xcc*-associated genes** | | | | | |
| 6 | AvrBs2 (AAY47144.1) | *X. campestris* pv. *campestris* | 100 | 100 | 0 |
| | AvrBs2 (AAM39371.1) | *X. campestris* pv. *campestris* | 100 | 100 | 0 |
| | AvrBs2 (CAP49385.1) | *X. campestris* pv. *campestris* | 100 | 100 | 0 |
| | AvrBs2 (CAJ21683.1) | *X. campestris* pv. *vesicatoria* | 99 | 77 | 0 |
| | AvrBs2 (BAE66903.1) | *X. oryzae* pv. *oryzae* | 100 | 76 | 0 |
| 7 | XopAM (CAP52621.1) | *X. campestris* pv. *campestris* | 100 | 100 | 0 |
| | XopAM (AAY50204.1) | *X. campestris* pv. *campestris* | 99 | 100 | 0 |
| | XopAM (AAM40388.1) | *X. campestris* pv. *campestris* | 98 | 100 | 0 |
| 8 | XopK (CAP50604.1) | *X. campestris* pv. *campestris* | 100 | 100 | 0 |
| | XopK (AAY48279.1) | *X. campestris* pv. *campestris* | 98 | 100 | 0 |
| | XopK (AAM42171.1) | *X. campestris* pv. *campestris* | 98 | 100 | 0 |
| | XopK (CAJ24946.1) | *X. campestris* pv. *vesicatoria* | 99 | 57 | 0 |
| | XopK (AAW75022.1) | *X. oryzae* pv. *oryzae* | 99 | 58 | 0 |
| 9 | XopQ (AAM40371.1) | *X. campestris* pv. *campestris* | 97 | 100 | 0 |
| | XopQ (CAP52639.1) | *X. campestris* pv. *campestris* | 97 | 99 | 0 |
| | XopQ (AAY50221.1) | *X. campestris* pv. *campestris* | 97 | 99 | 0 |
| | XopQ (ZP_02241198.1) | *X. oryzae* pv. *oryzicola* | 84 | 64 | 4.48E-170 |
| | XopQ (AAW77720.1) | *X. oryzae* pv. *oryzae* | 83 | 65 | 1.57E-169 |
| 10 | No hit | - | - | - | - |
| 11 | No hit | - | - | - | - |
| 12 | No hit | - | - | - | - |

Abbreviations: *Xcr*, *Xanthomonas campestris* pv. *raphani*; *Xcc*, *X. campestris* pv. *campestris*.

[a] The gene sequence used in BLASTX searches is described in Table 30 (Section 5.3.2, Chapter 5).

# APPENDIX 19. Breadth of coverage of avirulence-associated pan-gene sequences from alignments of genomic sequence reads of strains of *Xanthomonas campestris* pathovars *raphani* and *campestris*

The avirulence-associated pan-genes detected in races of *X. campestris* pv. *raphani* and *X. campestris* pv. *campestris* were selected following the predicted presence/absence patterns of avirulence genes in races of these pathovars shown in Table 27 (Section 5.1, Chapter 5). See details for the criteria used for selection of each pan-gene in Sections 5.2.6 and 5.3.3 (Chapter 5).

| Strain | | Pan-gene no. (avirulence gene) | | | |
|---|---|---|---|---|---|
| **Race** | **Accession** | 13 (A1r) | 14 (A1r) | 15 (A2r and A3c) | 16 (A1c) |
| ***Xanthomonas campestris* pv. *raphani*** | | | | | |
| 1 | HRI6490 | 98.6 | 98.4 | 0.0 | 0.0 |
| 1 | HRI8298 | 98.6 | 98.1 | 0.0 | 0.0 |
| 1 | HRI8803 | 98.6 | 98.1 | 0.0 | 0.0 |
| 2 | HRI6520 | 0.0 | 0.0 | 98.6 | 98.6 |
| 2 | HRI8305 | 0.0 | 0.0 | 98.6 | 0.0 |
| 2 | HRI8474 | 0.0 | 0.0 | 98.6 | 0.0 |
| 3 | HRI6519 | 0.0 | 0.0 | 0.0 | 0.0 |
| 3 | HRI8299 | 0.0 | 0.0 | 0.0 | 0.0 |
| 3 | 756 | 0.0 | 0.0 | 0.0 | 98.6 |
| ***X. campestris* pv. *campestris*** | | | | | |
| 1 | HRI3811 | 0.0 | 64.3 | 0.0 | 98.6 |
| 1 | HRI6195 | 0.0 | 65.0 | 0.0 | 98.6 |
| 1 | HRI8333 | 0.0 | 64.8 | 0.0 | 98.6 |
| 1 | B100 | 0.0 | 64.3 | 0.0 | 98.6 |
| 2 | HRI3849A | 0.0 | 55.2 | 0.0 | 6.6 |
| 3 | HRI8819 | 0.0 | 97.4 | 98.6 | 0.0 |
| 3 | ATCC33913 | 0.0 | 0.0 | 98.6 | 98.6 |
| 4 | HRI1279A | 0.0 | 98.1 | 0.0 | 98.5 |
| 4 | HRI6312A | 4.5 | 97.7 | 8.5 | 98.6 |
| 4 | HRI7758 | 0.0 | 97.9 | 0.0 | 98.6 |
| 4 | HRI7806 | 0.0 | 98.4 | 0.0 | 98.6 |
| 4 | HRI8506 | 0.0 | 97.9 | 0.0 | 98.6 |
| 4 | HRI8821 | 0.0 | 98.4 | 0.0 | 98.6 |
| 5 | HRI3880 | 42.2 | 98.4 | 98.6 | 0.0 |
| 5 | HRI6382 | 0.0 | 0.0 | 98.6 | 8.3 |
| 6 | HRI6181 | 98.6 | 48.8 | 0.0 | 0.0 |
| 6 | HRI6185 | 98.6 | 47.9 | 25.2 | 14.6 |
| 6 | HRI8450B | 0.0 | 98.4 | 0.0 | 0.0 |
| 6 | HRI8497 | 98.6 | 65.3 | 0.0 | 0.0 |
| 6 | HRI8806 | 0.0 | 98.4 | 0.0 | 0.0 |
| 7 | HRI8450A | 0.0 | 98.1 | 0.0 | 0.0 |
| 8 | HRI8815 | 0.0 | 97.9 | 98.6 | 98.6 |
| 9 | LMG8004 | 0.0 | 0.0 | 98.6 | 98.6 |

# APPENDIX 20. BLASTX top five hits plus the best hit to any type III effector, of candidate avirulence gene sequences against the RefSeq protein database of NCBI

| Gene no. [a] | BLASTX hits | | | | |
|---|---|---|---|---|---|
| | **Protein name (RefSeq accession)** | **Organism name** | **Query coverage (%)** | **Identity (%)** | **E-value** |
| 13 | AAA family ATPase (WP_052844664.1) | *Xanthomonas campestris* | 99 | 100 | 0 |
| | AAA family ATPase (WP_050495059.1) | *X. campestris* | 99 | 99 | 0 |
| | ATPase AAA (WP_046966406.1) | *Xanthomonadales* | 99 | 74 | 6.00E-129 |
| | AAA family ATPase (WP_056145618.1) | *Duganella* sp. | 91 | 73 | 1.00E-118 |
| | AAA family ATPase (WP_061178981.1) | *Burkholderia* sp. | 97 | 70 | 5.00E-117 |
| 14 | Hypothetical protein (WP_054088409.1) | *X. campestris* | 99 | 98 | 1.00E-97 |
| | Hypothetical protein (WP_043889248.1) | *X. hortorum* | 99 | 97 | 9.00E-97 |
| | Hypothetical protein (WP_016903205.1) | *X. arboricola* | 90 | 86 | 9.00E-66 |
| | Hypothetical protein (WP_006449414.1) | *X. gardneri* | 99 | 89 | 8.00E-86 |
| | Hypothetical protein (WP_056664642.1) | *Rhizobacter* sp. | 96 | 47 | 4.00E-32 |
| | Type III effector protein (WP_028854687.1) | *Ralstonia solanacearum* | 72 | 36 | 4.00E-11 |
| 15 | Hypothetical protein (WP_011038360.1) | *X. campestris* | 99 | 100 | 4.00E-149 |
| | Type III effector HopH1 (WP_054393958.1) | *X. vasicola* | 96 | 41 | 2.00E-33 |
| | Type III effector HopH1 (WP_017115174.1) | *X. vasicola* | 96 | 42 | 2.00E-32 |
| | Type III effector HopH1 (WP_039440092.1) | *X. vasicola* | 96 | 40 | 7.00E-31 |
| | Hypothetical protein (WP_061274318.1) | *Cedecea neteri* | 98 | 40 | 2.00E-29 |
| 16 | Short-chain dehydrogenase/reductase (WP_039417818.1) | *X. euvesicatoria* | 97 | 99 | 0 |
| | Short-chain dehydrogenase/reductase (WP_008575517.1) | *X. perforans* | 97 | 98 | 0 |
| | Short-chain dehydrogenase/reductase (WP_046932214.1) | *X. perforans* | 97 | 98 | 0 |
| | Short-chain dehydrogenase/reductase (WP_041855016.1) | *X. euvesicatoria* | 97 | 98 | 0 |
| | Short-chain dehydrogenase/reductase (WP_046933490.1) | *X. perforans* | 97 | 98 | 0 |

[a] The gene sequence used in BLASTX searches is described in Table 33 (Section 5.3.3, Chapter 5).

## APPENDIX 21. BLASTX top five hits of candidate avirulence gene sequences against predicted/known type III effector protein sequences of *Xanthomonas* spp. (listed in the *Xanthomonas* Resource database, http://www.xanthomonas.org/t3e.html)

| Gene no. [a] | BLASTX top hit | | | | |
|---|---|---|---|---|---|
| | **Type III effector (GenBank accession)** | **Organism name** | **Query coverage (%)** | **Identity (%)** | **E-value** |
| 13 | No hit | - | - | - | |
| 14 | No hit | - | - | - | |
| 15 | XopG (AAM42528.1) | *X. campestris* pv. *campestris* | 100 | 100 | 3.68E-154 |
| | XopG (AAY48041.1) | *X. campestris* pv. *campestris* | 47 | 99 | 1.03E-66 |
| | XopG (CAP52016.1) | *X. campestris* pv. *campestris* | 99 | 40 | 3.68E-35 |
| | XopG (CAJ22929.1) | *X. campestris* pv. *vesicatoria* | 99 | 40 | 3.93E-35 |
| | XopG (BAE71013.1) | *X. oryzae* pv. *oryzae* | 67 | 46 | 3.43E-30 |
| 16 | No hit | - | - | - | |

[a] The gene sequence used in BLASTX searches is described in Table 33 (Section 5.3.3, Chapter 5).